COMPARATIVE ANALYSIS OF MICROSPORIDIA OF FIRE ANTS, SOLENOPSIS RICHTERI AND S. INVICTA

By

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To my dear family and friends

Iucundi Acti Labores

O Son of Spirit!
My first counsel is this: Possess a pure,
kindly and radiant heart, that thine may be a
sovereignty ancient, imperishable
and everlasting.

Bahá'u'lláh
The Hidden Words

Go to the ant, thou sluggard; consider her way and be wise:
Which having no guide, overseer, or ruler,
Provideth her meat in the summer,
And gathereth her food in the harvest.

The Holy Bible
Proverbs 6,6

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LIST OF ABBREVIATIONS

ASP	Ammonium persulfate
BSA	Bovine serum albumine
Clustal	Software program for multiple alignment of sequences
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
FAME	Fatty acid methyl ester
GAP	Software program to make optimal alignment between two sequences by inserting gaps to maximize the number of matches
GC	Gas chromatography
GCG	Genetics Computer Group
GC-MS	Gas chromatography- mass spectrometry
GTE	Glucose,Tris, EDTA buffer
LB	Luria-Bertani
MAP	Software program to display both strands of a DNA sequence with a restriction map shown above the sequence
MIDI	Microbial ID Inc.
MIS	Microbial Identification System
PAUP	Phylogenetic analysis using parsimony
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PileUp	Software program for multiple alignment of sequences
pTZ 19R	Bacterial plasmid
RFLP	Restriction fragment length polymorphism

rDNA	ribosomal RNA gene
RNA	Ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA
SDS	Sodium dodecyl sulfate
S.O.C.	Superoptimal catabolite (bacterial medium)
SSC	Standard saline citrate
STE	Sodium chloride, Tris, EDTA buffer
TAE	Tris-acetate, EDTA buffer
TB	Terrific broth
TBE	Tris-borate, EDTA buffer
TE	Tris, EDTA buffer
TEM	Transmission electron microscopy
TEMED	N, N, N', N' - tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
USDA-ARS	United States Department of Agriculture-Animal Research Service
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract of Dissertation Presented to the Graduate School
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COMPARATIVE ANALYSIS OF MICROSPORIDIA OF FIRE ANTS, *SOLENOPSIS RICHTERI* AND *S. INVICTA*

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Major Department: Entomology and Nematology

Two dimorphic microsporidia, *Thelohania* sp. and *Vairimorpha* sp., occur simultaneously in the black imported fire ant, *Solenopsis richteri*, in parts of Argentina and are considered as biological control agents of *S. invicta* in the United States. On the light-microscopic level, they are indistinguishable from *T. solenopsae* and *V. invictae*, described from Brazilian *S. invicta*. Two questions arise: Are *Vairimorpha* sp. and *Thelohania* sp. different phenotypes of the same species? Are *Thelohania* sp. and *Vairimorpha* sp. conspecific with *T. solenopsae* and *V. invictae*, respectively?

Morphological analysis revealed that spore dimensions and ultrastructures of *Thelohania* sp. and *Vairimorpha* sp. are comparable to those of *T. solenopsae* and *V. invictae*, respectively. The application of FAME profiles for the identification of microsporidia was assessed for the first time, using spores of *Thelohania* sp., *Nosema algerae*, and *Vairimorpha necatrix*. Even though the three species had qualitatively and quantitatively different FAME profiles, this method was unsuitable for characterization of

the remaining fire ant microsporidia because of (1) influence of host insect on FAME profile and (2) requirement of large sample sizes ($1x10^9$ spores) for FAME analysis.

PCR products of the 16S rRNA gene of Thelohania sp. and T. solenopsae were the same at ~ 1,400 bp as compared to that of Vairimorpha sp. at ~ 1,300 bp. V. invictae could not be included in the genotypic analysis because of sample size limitations. Restriction analysis of the PCR products with several enzymes differentiated Vairimorpha sp. from Thelohania sp. and T. solenopsae which were not separable from each other. Sequence analysis of the 16S rRNA gene of T. solenopsae, Thelohania sp., and Vairimorpha sp. showed that the two Thelohania species have a very high sequence similarity amongst each other (> 99%). Vairimorpha sp. has a 63% sequence similarity with T. solenopsae and Thelohania sp.

In conclusion, the available phenotypic and genotypic data support the hypothesis that Thelohania sp. and Vairimorpha sp. are not different phenotypes of the same species but separate species. Thelohania sp. and T. solenopsae appear to be conspecific and probably represent two subspecies. V. invictae and Vairimorpha sp. appear indistinguishable morphologically but await genotypic analysis.

CHAPTER I
TAXONOMIC PROBLEMS OF FIRE ANT MICROSPORIDIA

Synopsis

The red imported fire ant, *Solenopsis invicta*, is a major agricultural and urban pest in the southeastern United States (Stimac and Alves 1994; Patterson 1990; Adams 1986). Despite extensive primarily chemical control efforts, it is firmly established in the southeastern United States (Stimac and Alves 1994). Nest density of red imported fire ants is much higher in the United States than in its native South America, and *S. invicta* constitutes a much larger fraction of the ant community in the US than in South America (Porter et al. 1992). The very successful colonization of the southeastern US by *S. invicta* may be in part attributed to the fact that it faces virtually no natural biological control in the US (Buren et al. 1978, 1983). Natural enemies of fire ants are extremely rare in the US but abundant in South America (Jouvenaz 1983; Stimac and Alves 1994).

In an effort to find a good biological control agent for *S. invicta* in the US, several surveys for natural enemies have been conducted in South America (summarized by Jouvenaz 1983; Stimac and Alves 1994). The microsporidium *Thelohania solenopsae* is the first specific pathogen described from the red imported fire ant, *S. invicta* in Brazil (Allen and Buren 1974). Subsequently, another microsporidium, *Vairimorpha invictae*, was detected in *S. invicta* in Brazil (Jouvenaz and Ellis 1986). Briano (1993) reported a high incidence of infection of the black imported fire ant *S. richteri* from Argentina with *T. solenopsae*-like and *V. invictae*-like microsporidia, hereforth called *Thelohania* sp. and *Vairimorpha* sp. *Thelohania* sp. and *Vairimorpha* sp. may occur in dual infections in the same individual ant. The infections appear to weaken the ant colonies and reduce total numbers of ants significantly (Briano 1993; R.S. Patterson, personal communication).

Solenopsis richteri and S. invicta were considered to be different color morphs of one species, S. saevissima richteri Forel (Wilson 1951) until Buren (1972) described S. invicta and S. richteri as separate species. Solenopsis invicta interbreeds successfully with S. richteri in areas of the US where their ranges overlap, and taxonomy of the two ant species is still not resolved (Vander Meer and Lofgren 1986). The microsporidia found in the Argentinean S. richteri could be introduced into the US as biological control agents after several taxonomic and ecological studies are completed.

The research presented here will address the following taxonomic questions: Are Thelohania sp. and Vairimorpha sp. two different phenotypes of the same species or are they indeed different species? Are T. solenopsae and Thelohania sp. and V. invicta and Vairimorpha sp., respectively, conspecific or are they separate species?

Traditionally, microsporidian taxonomy and classification has been based on spore morphology, life cycles and host specificities. Characterization based solely upon simple morphology can result in misleading classification, because spores of different microsporidian species may appear to be phenotypically identical. For example, two species of microsporidia, Encephalitozoon hellem and E. cuniculi, isolated from AIDS patients, can be differentiated using biochemical and immunological tests, but not by fine structure or development (Didier et al. 1991). Furthermore, one species may have several different spore phenotypes, depending on host and life stage. Microsporidia requiring an intermediate host express distinct spore phenotypes in the intermediate and definite hosts (Andreadis 1985; Becnel 1992; Sweeney et al. 1985), whereas those which develop in only one host may also be heterosporous (Becnel et al. 1989). Environmental factors, such as temperature, can affect the expression of different spore phenotypes (Jouvenaz and Lofgren 1984). Incomplete understanding of the often complex life cycles involving several hosts, and spore types, also hampers experimental transmission of many species in the laboratory.

Molecular techniques, including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and sequence alignment of the small 16S rRNA subunit, are being developed for microsporidian species identification and phylogenetic construction (Baker et al. 1995, Baker et al. 1994; Weiss et al. 1994; Vossbrinck et al. 1993). Additional methodologies, including spore protein profiles (Didier et al. 1991; Irby et al. 1986; Jahn et al. 1986; Langley et al. 1987; Street 1976), serological assays (Canning 1988; Didier et al. 1991; Niederkorn et al. 1980; Oien and Ragsdale 1992), and flow cytometry (Amigo et al.1994) have been used to aid in classification, although to a limited extent.

In this study, a multiphasic approach was used to compare the micrososporidia from S. richteri to each other and to those from S. invicta. Methods used included light microscopic and ultrastructural observations of the spores (chapter II), and amplification, sequencing, and sequence comparison of the 16S rRNA genes (chapter IV). In addition, the use of spore fatty acid profiles was investigated for the first time as a character in identification (chapter III).

CHAPTER II
MORPHOLOGICAL CHARACTERIZATION OF MICROSPORIDIA FROM SOLENOPSIS INVICTA AND S. RICHTERI

Introduction

Fire ants belong to the genus Solenopsis. Six species -- four native and two introduced -- and two hybrids occur in North America. Solenopsis geminata (Fabricius), S. xyloni (MacCook), S. amblychila Wheeler, and S. aurea Wheeler are native species and found in the southern states (Trager 1991). As indicated in their names, the black imported fire ant, S. richteri Forel, and red imported fire ant, S. invicta Buren, are not native to this country but were introduced from South America. Two hybrid forms, S. xyloni x geminata and S. richteri x invicta, occur as well in the United States (Trager 1991).

Solenopsis invicta is common in the southwestern region of Brazil (Pantanal, a large flood plain of the head waters of the Paraguay river) westward through Rhondonia and southward along the Paraguay River through Bolivia to the northern border of Argentina with Paraguay and Uruguay (Buren et al. 1974). Solenopsis richteri is prevalent in the more temperate southern states of Brazil, Uruguay and Argentina. Lofgren (1986) summarizes in great detail the early history of imported fire ants in the United States. Briefly, S. richteri was introduced into Mobile, Alabama, around 1918 (Creighton 1930) and is now established in areas of northeastern Mississippi and northwestern Alabama. The red imported fire ant, S. invicta, reached Mobile, Alabama, in the 1940s (Wilson and Eads 1949) and subsequently spread throughout the southeastern states displacing S. richteri everywhere except in pockets of northeastern Mississippi and northwestern Alabama. Originally, S. invicta and S. richteri were considered different color morphs of the same species, S. saevissima richteri Forel (Wilson 1951). Buren (1972) was the first one to describe S. invicta as a separate species. S. invicta and S. richteri interbreed in areas of Alabama, Mississippi, and Georgia where their ranges overlap (Ross et al. 1987; Vander Meer and Lofgren 1988). Since the two species mate and are successful at producing viable hybrids, the validity of S. invicta as a separate species is questionable but recognized as such until further investigations (Vander Meer and Lofgren 1988). Imported fire ants are considered agricultural and urban pests in the United States and represent a human health risk. They are generally no problem in South America (Stimac and Alves 1994; Patterson 1993; Adams 1986).

Imported fire ant populations occur at relatively low levels in their South American homelands. Fire ant mound densities are much higher in the US than in Brazil, and fire ants constitute a much larger fraction of the ant community in the US (Porter et al. 1992). Reasons for this could be lack of predators, pathogens, and competitors of imported fire ants in the US (Buren et al. 1978; 1983). Stimac and Alves (1994) present a summary on red imported fire ant ecology in their homelands and the US. Further summaries can be found in Lofgren and Vander Meer's book on fire ants and leaf-cutting ants (1986).

Only a few surveys for pathogens of fire ants in the United States have been conducted. Jouvenaz et al. (1977) have used a screening method sufficiently sensitive to detect low levels of microsporidian and fungal infections but not bacteria and viruses. They find no pathogens in S. richteri; in S. invicta they detect very low levels of a benign, unidentified yeast infection, and only 1 in 1007 colonies has an unidentified microsporidian disease. This microsporidium is found in low levels in S. geminata (Fabricius) colonies. Subsequently, Jouvenaz and Hazard (1978) described Burenella dimorpha , a new microsporidian genus and species, from S. geminata and created a new family. Beckham and Bilimoria (1982) examined samples of 113 ant species including S. invicta from western Texas for the presence of fungi, occluded viruses, microsporidia, and nematodes and found resting spores of Entomophthora sp. on only one specimen of Pheidole bicarinata vinelandica Forel. No parasites or pathogens are found in fire ants. Jouvenaz and Kimbrough (1991) described an endoparasitic fungus, Myrmecomyces annellisae gen.nov., sp. nov., from Solenopsis quincecuspis Forel, collected in Buenos Aires Province, Argentina, and S. invicta, collected in Florida. Gross pathology, histopathology, or changes in host behavior are not observed but parasitized hosts appear to succumb more readily to stress. Two fungi, Conidiobolus sp. and Metarhizium anisopliae, were observed by Sanchez-Peña and Thorvilson (1992) on S. invicta queens collected in Texas. Of the adult queens and workers, and worker larvae assayed against conidial showers of Conidiobolus sp., only the worker larvae die. Metarhizium anisopliae kills challenged alate workers.

The sparse occurrence of natural enemies of imported fire ants in the United States is a good example of the introduction of an insect into another country without its predators, parasites and pathogens. A parallel situation occurred with the gypsy moth, Lymantria dispar. This insect was introduced into the United States from Europe where it has numerous natural enemies which are lacking in the United States (Howard and Fiske 1911). With regard to pathogens, one major group of pathogens, the microsporidia, have been identified as significant mortality factors in Eurasian gypsy moth populations but they have not been recorded from gypsy moths in North America (Jeffords et al. 1989). Microsporidia isolated from European L. dispar are evaluated as biocontrol agents for the US populations (Maddox et al. 1992).

Numerous reports on natural enemies of S. richteri and S. invicta in their homelands Argentina, Brazil, and Uruguay include microsporidia, fungi, nematodes, parasitic wasps and flies and are summarized by Stimac and Alves (1993) and Jouvenaz (1983). Documented parasites include Orasema wasps (Heraty et al. 1993), the straw itch mite Pyemotes tritici (Thorvilson et al. 1987), an unidentified phorid fly (Wojcik et al. 1987), the phorid Pseudacteon obtusus (Williams and Banks 1987), the nematode Tetradonema solenopsis (Nickle and Jouvenaz 1987), and unidentified nematodes

(Jouvenaz and Wojcik 1990). Pathogens include a virus, neogregarines, microsporidia, a bacterium (Jouvenaz 1983) and the entomopathogenic fungi Beauveria, Metarhizium and Paecilomyces (Stimac et al. 1987). Recently, a high incidence of infection of S. richteri with Thelohania solenopsae-like and Vairimorpha invictae-like microsporidia, hereforth called Thelohania sp. and Vairimorpha sp., was reported from Argentina (Briano 1993). He found that in areas where the microsporidia are present the density of fire ant mounds is significantly lower than in uninfected areas. These microsporidia are currently being evaluated as potential biological control agents for imported fire ants in the United States.

Thelohania solenopsae, the first specific pathogen known from fire ants, was first reported by Allen and Buren (1974) from S. invicta in Brazil. They detected spore cysts formed from enlarged fat body cells in the gasters of alcohol-preserved workers. Giemsa-stained smears of fat body tissue of live workers show octonucleate sporonts that produce eight spores enclosed in a sporophorous vesicle. Based on this characteristic they identified it as a species of the genus Thelohania Henneguy but they did not give it a species name. The genus Thelohania was defined by Henneguy and Thélohan (1892) as having spores of only one developmental sequence, which produces octospores enclosed in a sporophorous vesicle or interfacial envelope. Knell and Allen (1977) described Thelohania sp. from live S. invicta workers and brood in Brazil as a new species, T. solenopsae. Meronts (vegetative stages) are found in fat body of larvae, pupae and queen ovaries (Knell and Allen 1977). Sporonts occur in late pupae, workers, males and queens. Spores occur only in adults. Knell and Allen (1977) discovered that T. solenopsae is dimorphic producing both bacilliform binucleate free spores and pyriform uninucleate octospores in the same individuals. This makes it the only dimorphic member of the genus Thelohania. However, the type species, T. giardia, which has not been carefully studied (Hazard and Oldacre 1975), may also be dimorphic.

Allen and Silveira-Guido (1974) also found Thelohania sp. in workers of S. richteri in Montevideo, Uruguay and Las Flores, Argentina and from a Solenopsis sp. in

Montevideo. They speculated, based on their knowledge of S. invicta and its microsporidian parasites, that the ant populations are regulated naturally by the microsporidia. It is unclear whether T. solenopsae is one species or a complex of sibling species of microsporidia (Jouvenaz 1986) since it has been detected in more than a dozen described or undescribed Solenopsis spp. in South America (Jouvenaz 1983).

The dimorphic V. invictae has been reported from S. invicta collected in Brazil by Jouvenaz and Ellis (1986). Vegetative stages are found in larvae and pupae, free spores in pupae and adults, and octospores in adults. The genus Vairimorpha was created by Pilley (1976) to include dimorphic species with disporous and octosporous sporogony in the same individual.

The classification of T. solenopsae and V. invicta may have to be revised (Jouvenaz and Ellis 1986). Knell and Allen (1977) placed T. solenopsae in the family Thelohaniidae because it meets all the family criteria. It produces octospores and sporoblasts by endogenous budding, and it secretes metabolic products retained by the sporophorous vesicle. They placed it in the genus Thelohania because of its isofilar polar filament. Two genera of Thelohaniidae at that time, Amblyospora and Parathelohania, produce both octospores and free spores, but free spores arise from plasmodia (4-40 spores per plasmodium, multisporous sporogony) (Hazard and Oldacre 1975). The free spores of T. solenopsae, however, arise from diplokaryotic sporonts (disporous sporogony) which is characteristic of Vairimorpha. Jouvenaz and Hazard (1978) created the family Burenellidae for species having two sporogonic sequences, one producing free spores from disporous sporogony, and the other producing meiospores from octonucleate sporonts. Species of Burenellidae also develop tubules within the sporophorous vesicle during sporulation, they do not secrete granules as do those of the Thelohaniidae. The genera Burenella, Vairimorpha, Evlachovaia, and Pilosporella are included in Burenellidae (Sprague et al. 1992). It appears that T. solenopsae does not quite fit into the family Thelohaniidae because it produces free spores from disporous sporonts and not from plasmodia. Furthermore, the species of this family are common parasites of a variety of aquatic and semiaquatic animals. Thelohania solenopsae does not quite fit into the Burenellidae either, because it does secrete granules during octosporous sporulation.

Based on light microscopic observations, Thelohania sp. and Vairimorpha sp. from Argentine S .richteri appear to be identical to T. solenopsae and V. invictae from Brazilian S. invicta. The objective of this study was to compare light- and ultrastructural features of Argentine Thelohania sp. and Vairimorpha sp. and Brazilian T. solenopsae and V. invictae.

Infected S. invicta and S. richteri were collected by R.S. Patterson and J. Briano in Brazil and Argentina. Identity of the ants from which the microsporidia were isolated for all experiments was confirmed by determination of cuticular hydrocarbon components with gas chromatography (GC). Both S. invicta and S. richteri have signature cuticular hydrocarbon profiles which clearly define the two species (Vander Meer and Lofgren 1988; Nelson et al. 1980).

Materials And Methods

Collection and Processing of Ants

Thelohania sp. and Vairimorpha sp. were obtained from S. richteri adults collected by R. S. Patterson and J. Briano in the area of Saladillo, Buenos Aires province, 180 km SW of Buenos Aires in Argentina. R. S. Patterson and J. Briano also collected S. invicta infected with T. solenopsae and V. invictae in the area of Cuiaba, Brazil. The ants were transported back to Gainesville, Florida, in artificial ant nests modified from Williams (1989). To make a 'nest', powdered dental labstone (roughly 250 g) and tapwater were thoroughly mixed to get a thick liquid paste. The labstone paste was then poured into the bottom of a large petri dish (150 x 25 mm) and allowed to harden. Prior to use it was saturated with water to maintain a high relative humidity inside the petri plate. A small test tube filled with 1 M aqueous sucrose solution and plugged with cotton gauze was secured inside the petri dish lid. For transport, the ants were confined within the modified petri dish with the lid taped securely to the bottom. On their arrival to Gainesville, the ants were either frozen at -70°C to purify the spores later or processed immediately.

Some colonies of infected S. richteri were maintained over several months in the laboratory. For rearing of the ant colonies, big trays (95 x 78 x 28 cm) were coated with Fluon on the inside walls to prevent ants from escaping. Each tray had one artificial ant nest, the construction of which is described in detail by Williams (1989). In principle, the ant nest was like the one described above except that a small petri dish (100 x 10 mm) with four small holes in the bottom, containing a sponge and Tygon tubing inserted in a U-shape through two holes in the top of the small petri-dish, was placed into the large petri dish. The lab stone was poured into the larger dish, leaving only the tubing exposed. The purpose of the small dish with the sponge was to act as water reservoir to keep the ant nest humid. One colony was placed in each tray. Food consisted of frozen crickets, honey agar, 1 M aqueous sucrose and water. The rearing temperature was about 23°C. Poor collection conditions in Brazil yielded only very few S. invicta; thus no colonies of S. invicta could be established in the laboratory. Thelohania solenopsae, Thelohania sp. and Vairimorpha sp. but no V. invictae could be isolated from the available specimens.

Ant Identification

To extract cuticular hydrocarbons, five worker ants, frozen at -20°C until analysis, were soaked in 1 mL of hexane in small vials for 2 h. The total lipid extract was passed through a short column (3 cm long x 0.5 cm in diameter) of silicic acid (60-200 mesh, J.T. Baker, Philadelphia, Pa.) in a Pasteur pipette (Carlson and Bolten 1984). To make a column, a little glass wool was stuffed into a Pasteur pipette and the silica gel poured on top of it. The hydrocarbons were eluted from the mini-column with 3 mL of hexane, concentrated to dryness with nitrogen gas, and redissolved in 20 μL of hexane for GC analysis (Carlson and Brenner 1988). Oxygenated compounds, if present, remained on the column.

Gas chromatography analyses of hydrocarbons were conducted using a 5890 series II Hewlett Packard gas chromatograph with a flame ionization detector. The column oven was fitted with a 30 m x 032 mm i.d. x 0.25 μm film thickness fused silica capillary column of DB-1. Following a cool-on column injection of 1 μL at 63°C, the oven temperature was raised to 230°C at 25°C/min, and then to a final temperature of 320°C at 7°C/min. The temperature was held at 320°C for 15 min. The carrier gas was hydrogen. The data were processed by HP Chemstation, version 1.0 software.

Phase Contrast Microscopy

Diagnosis of infection was made by examining wet mounts of fat body tissue of adults by phase contrast microscopy. Different body parts were examined for infection (head, thorax, gaster). Fresh samples of Thelohania sp. and Vairimorpha sp. were used for spore measurements with a calibrated Vickers image-splitting micrometer.

Transmission Electron Microscopy

Spore cysts of Thelohania sp., Vairimorpha sp., and T. solenopsae were dissected from adult worker ant gasters in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% $CaCl_2$. After 30 min, the hardened cysts were transferred to fresh 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% $CaCl_2$ and fixed for 2-4 h at room temperature. They were postfixed in 1% aqueous OsO_4 (osmium tetroxide) (w/v) for 2 h at room temperature, dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite plastic (Mollenhauer 1964). Tissue blocks were thick-sectioned with a glass knife, and thin-sectioned with a diamond knife on a Sorvall ultramicrotome. Thin-sections were stained with methanolic uranyl acetate (50% methanol, 1% uranyl acetate) followed by lead citrate (Reynolds 1963). They were and photographed at an accelerating voltage of 75 kV with a Hitachi H-600 electron microscope.

Results

Ant Identification

Identification of the ants as either S. invicta or S. richteri was confirmed by their respective cuticular hydrocarbon profiles (Figure 2.1). In S. invicta, hydrocarbons with 28 and 29 carbons in the backbone of the molecules predominate whereas in S. richteri hydrocarbons with 24, 25, and 26 carbons in the backbone of the molecules predominate (Nelson et al. 1980).

Light Microscopy

Spores of Thelohania sp. and Vairimorpha sp. are found only in pupae and adults, not in the larval stages, of the ants. Workers, queens and males can be infected. The microsporidia seem to parasitize fat body cells which hypertrophy into spore-filled cysts. Dual infections with Thelohania sp. and Vairimorpha sp. in the same individual may occur. Thelohania sp. and T. solenopsae cause the development of comparatively large cysts while a Vairimorpha infection produces tiny cysts (Figure 2.2, Figure 2.3). The infection may be very heavy - 25 Thelohania sp. cysts and 14 Vairimorpha sp. cysts were counted from one major S. richteri worker! The infection produced symptoms not readily observed with a loss of coordination and slow movement of the diseased ants. Both Thelohania sp. and Vairimorpha sp. were dimorphic with uninucleate meiospores in groups of eight and binucleate free spores.

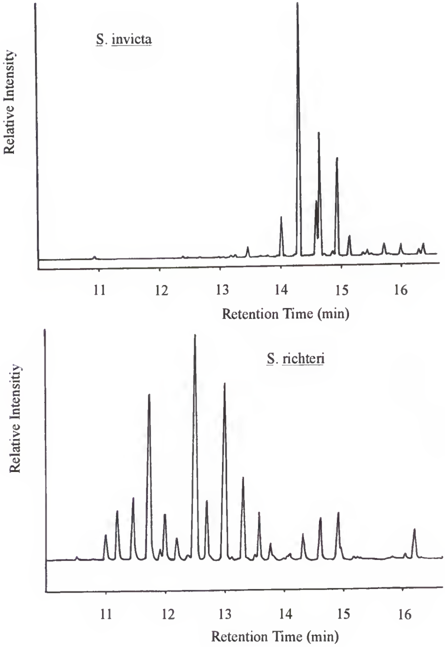
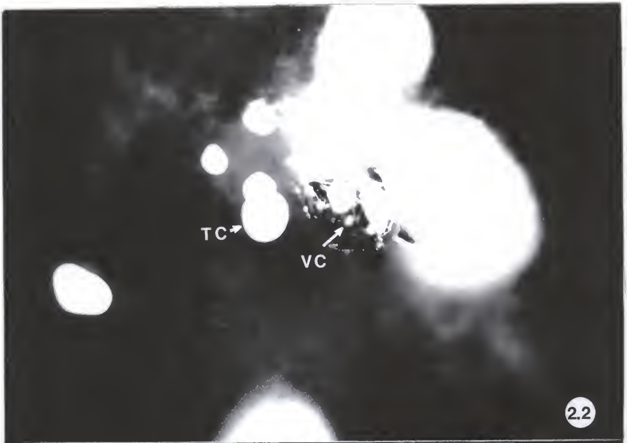


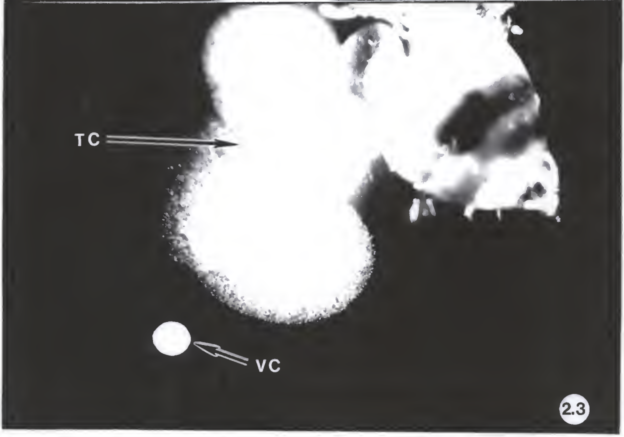
Figure 2.1. Gas chromatographic traces of S. invicta and S. richteri hydrocarbons. Ant lipids were extracted with hexane. The total lipid extracts were passed through short columns of silicic acid (3 cm long x 0.5 cm diameter) in Pasteur pipettes. The hydrocarbons were eluted from the columns with hexane in the void volume. They were analyzed with a 5890 series II Hewlett Packard gas chromatograph fitted with a fused silica capillary column of DB-1. A flame ionization detector was used.

Figure 2.2. Light micrograph of dissected S. richteri gaster with spore cysts of Vairimorpha sp. (VC) and Thelohania sp. (TC). x18.

Figure 2.3. Light micrograph of dissected S. richteri gaster with spore cysts of Vairimorpha sp. (VC) and Thelohania sp. (TC). x46.



2.2



2.3

Thelohania sp. was apparently confined to fat body tissue of ant gasters. Both uninucleate meiospores and binucleate free spores developed simultaneously in the same cysts. Octets of meiospores were enclosed into a persistent sporophorous vesicle (henceforth referred to as an interfacial envelope). Free spores were not enclosed in a membrane (Figure 2.4). Octospores are pyriform in shape and measure 2.32 ± 0.14 x 4.10 ± 0.31 µm (n=33). Free spores are elongate oval in shape and measure 2.83 ± 0.30 x 5.71 ± 0.55 µm (n=21). Free spores were exceedingly rare (usually less than 1%).

Vairimorpha sp. spores were observed from tissues within the head, thorax and gaster of S. richteri. In the gaster, fat body tissue was parasitized; the nature of the tissue in the thorax and head was unclear. Only mature free spores were found in pupae; both spore types occurred in the same cysts in adults (Figure 2.5). Similar to Thelohania sp., octets of meiospores were enclosed in interfacial envelopes, but the free spores were not enclosed in a membrane (Figure 2.6). Vairimorpha sp. spores are much larger than those of Thelohania sp. Octospores were ovoid and very slightly narrower at the anterior pole and measure 4.31 ± 0.25 x 6.45 ± 0.61 µm (n=33). Free spores were elongate bacilliform and measure 3.11 ± 0.19 x 10.76 ± 0.48 µm (n=34). Figure 2.7 shows Vairimorpha sp. and Thelohania sp. meiospores, Figure 2.8 all four spore types in the same field of view.

Transmission Electron Microscopy

Thelohania sp. meiospores were pyriform in sagittal section with the posterior end more broadly rounded than the anterior end. They were uninucleate with a lamellar polaroplast anteriorly and vacuole posteriorly. Polyribosomes border the nucleus. The polar filament was isofilar with 10-12 coils. The coils were arranged either uniform or irregular. The spore wall, composed of exospore and endospore, was relatively thin and undulating (Figures 2.9, 2.10). The polar filament consisted of several layers with

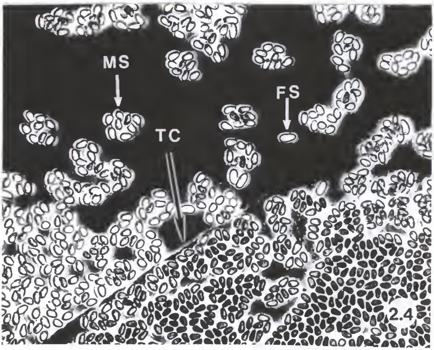
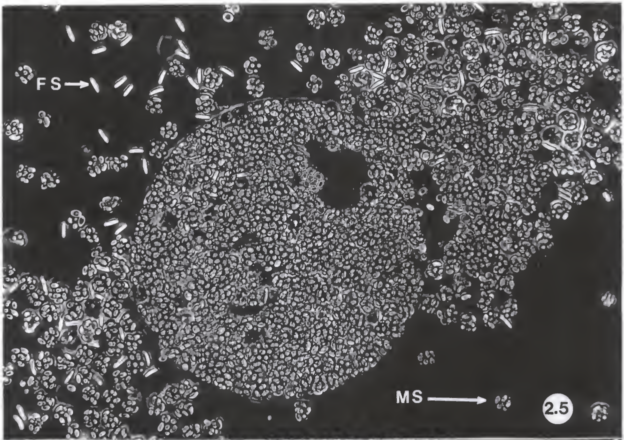
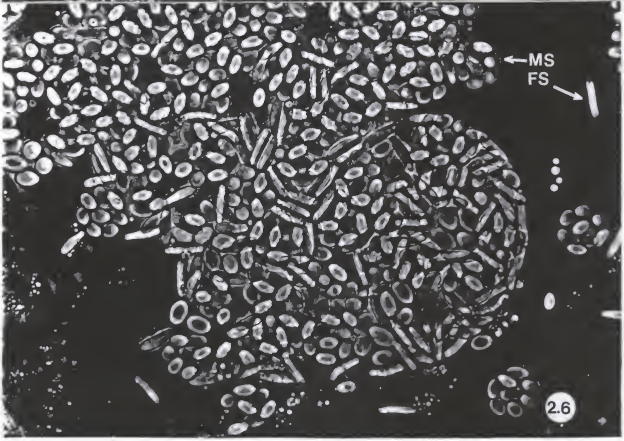


Figure 2.4. Light micrograph of Thelohania sp. partial cyst (TC) with meiospores (MS) and free spores (FS). x750.

Figures 2.5. Light micrograph of Vairimorpha sp. cyst with free spores (FS) and meiospores (MS). x210.

Figure 2.6. Light micrograph of Vairimorpha sp. cyst with free spores (FS) and meiospores (MS). x750.

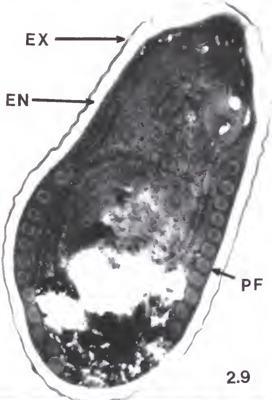

FS
MS
2.5


MS
FS
2.6

electron-dense outer layers and inner core separated by an electron-transparent zone (Figure 2.10). The exospore consisted of several layers and was about 1/5 the thickness of the electron-transparent endospore (Figure 2.10). Thelohania solenopsae meiospores looked similar to Thelohania sp. meiospores (Figure 2.11, 2.12).

Thelohania sp. free spores were ovoid in sagittal section. They were diplokaryotic with polyribosomes bordering the nuclei and a posterior vacuole. The polar filament was isofilar with 15 coils. It was composed of several layers. The spore wall was relatively thin and smooth with electron-dense exospore and electron-transparent endospore (Figure 2.13).

Vairimorpha sp. meiospores were pyriform in sagittal section. The most striking feature was the presence of an exceedingly thick smooth spore wall with a relatively thin electron-dense exospore and a very thick electron-transparent endospore (Figure 2.14). The polar filament was isofilar with 11-13 coils; the anterior polaroplast was lamellar (Figure 2.14). The exospore consisted of several layers and was about 1/9 the thickness of the endospore (Figure 2.15). The polar filament also consisted of several electron-dense and electron-transparent layers (Figure 2.15). The electron-transparent layer surrounding the electron-dense core was much thicker in Vairimorpha sp. than in Thelohania sp. Conversely, the electron-dense core was much smaller in Vairimorpha sp. than in Thelohania sp. Vairimorpha sp. free spores were very long and bacilliform in sagittal section. The polar filament was isofilar with ~ 26 coils. The polaroplast was lamellate. Since processing of these microsporidia for TEM is extremely difficult, good quality micrographs of the Vairimorpha sp. free spores could not be presented here.



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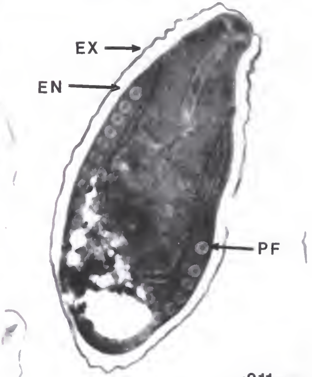

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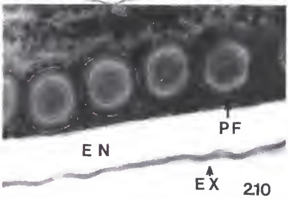

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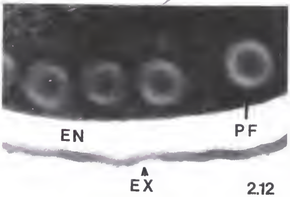


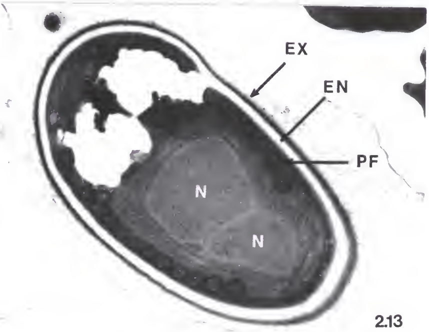
2.12

Figure 2.9. Electron micrograph of Thelohania sp. meiospore. x37,500.

Figure 2.10. Electron micrograph of Thelohania sp. spore wall and polar filament. x150,000.

Figure 2.11. Electron micrograph of T. solenopsae meiospore. x37,500.

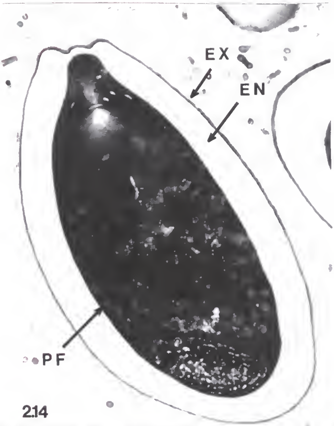
Figure 2.12. Electron micrograph of T. solenopsae spore wall and polar filament. Endospore (EN), exospore (EX), polar filament (PF). x150,000.



2.13

Figure 2.13. Electron micrograph of Thelohania sp. free spore; polar filament (PF), exospore (EX), endospore (EN). x30,000.

Figure 2.14. Electron micrograph of Vairimorpha sp. meiospore. x18,000.
Figure 2.15. Electron micrograph of Vairimorpha sp. meiospore spore wall and polar filament. x120,000. Endospore (EN), exospore (EX), polar filament (PF).



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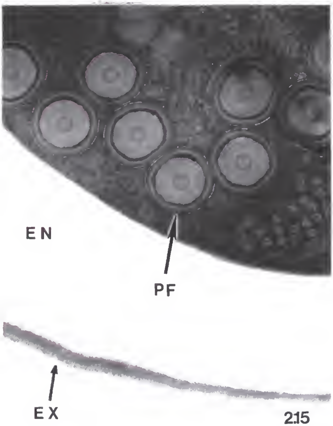


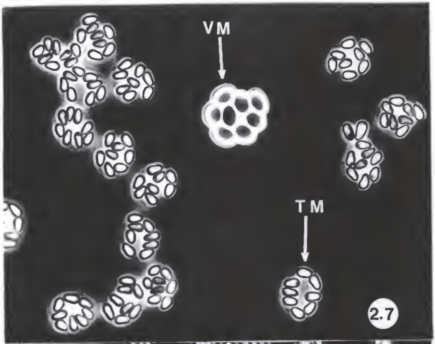
2.15

Discussion

Based on the morphological evidence Thelohania sp./T. solenopsae and Vairimorpha sp./V. invictae appear to be conspecific. Vairimorpha sp. and Thelohania sp. are very distinct from each other at the light-microscopic and ultrastructural level in both size and morphology. Thelohania sp. and Vairimorpha sp. are very similar to T. solenopsae and V. invictae, respectively, with regard to tissue specificity, and light and ultrastructural microscopy.

To determine whether Vairimorpha sp. and Thelohania sp. are different phenotypes of the same species, the possibilities of heterosporous microsporidia (Sweeney et al 1985; Becnel et al. 1989) and phenotypic plasticity must be considered. Even though Mitchell and Cali (1993) did not observe temperature-related differences in the ultrastructure of V. necatrix, there are reports of environmentally induced phenotype variation. Burenella dimorpha, a microsporidian parasite of S. geminata, for example, shows temperature-dependent spore dimorphism (Jouvenaz and Lofgren 1984). They demonstrated inhibition of octospore development at relatively low (20°C) and high temperatures (30°C). Temperature-dependent spore dimorphism has been demonstrated for other microsporidia as well (Maddox and Sprenkel 1978). This means that Vairimorpha sp. and Thelohania sp., even though ultrastructurally distinct, could be different phenotypes (expressed in different environmental conditions) of the same species.

Spore measurements of Thelohania sp. meiospores and free spores are about 1 µm larger than the previously published measurements of T. solenopsae by Knell and Allen (1977). This difference in spore dimensions could be due to a different technique to read the scale in the ocular micrometer (used for spore measurements) or to environmentally induced size variation due to temperature or host. For example, temperature regulated spore length of Vairimorpha sp. 696 (Sedlacek et al. 1985). Spores were significantly longer at 19°C (5.9 µm) than at 32°C (4.7 µm). Mean spore size of the same species of



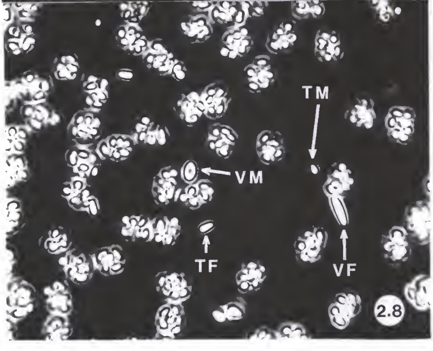


Figure 2.7. Light micrograph of meiospore octets of Thelohania sp. (TM) and Vairimorpha sp. (VM). x750.

Figure 2.8. Light micrograph of Thelohania sp. and Vairimorpha sp. free spores (TF, VF) and meiospores (TM, VM). x750.

microsporidia may also vary significantly with host species (Brooks and Cranford 1972). They found that spores of Nosema heliothidis are significantly shorter in Heliothis zea larvae than in its hymenopterous parasite Campoletis sonorensis (Brooks and Cranford 1972). Spore dimensions of Vairimorpha sp. meiospores and free spores are almost identical to the previously published dimensions of V. invictae (Jouvenaz and Ellis 1986). Furthermore, meiospore and free spore ultrastructure of Thelohania sp. and Vairimorpha sp. are very similar to those of T. solenopsae (Knell and Allen 1977) and V. invictae (Jouvenaz and Ellis 1986), respectively.

Meiospore ultrastructures of Thelohania sp. and T. solenopsae are characterized by a thin, undulating exospore, relatively thin endospore, lamellar polaroplast, and isofilar polar filament. Meiospore ultrastructures of Vairimorpha sp. and V. invictae are characterized by a very thick smooth spore wall with relatively thin exospore and very thick endospore, lamellar polaroplast, and isofilar polar filament. Differences were observed, however, in number of coils and arrangement of the coils of the polar filament. The number of turns of the polar filament, arrangement of the coils and number of broad and narrow coils of the meiospores may be used in distinguishing closely related species (Hazard and Oldacre1975). For example, Andreadis (1994) was able to distinguish six new species of the genus Amblyospora based upon distinct differences in the number of turns of the polar filament, arrangement of the coils and number of broad and narrow coils of the polar filament. Garcia and Becnel (1994) also utilized numerical ratio of broad and narrow coils, and arrangement of these coils as useful taxonomic characters to describe eight new species of microsporidia of the genera Amblyospora and Parathelohania from Argentine mosquitoes.

It was very difficult to quantify polar filament arrangement and number of coils in an adequate number of spores of Thelohania sp., T. solenopsae and Vairimorpha sp. because sample preparation for TEM was very difficult (see below). Based on the material available, Thelohania sp.meiospores showed both uniform and irregular arrangement of the coils of the polar filament but T. solenopsae meiospores had a uniform arrangement of coils of the polar filament. Thelohania sp. had between 10-12 coils whereas T. solenopsae had between 9-11 coils. Free spore ultrastructure, except for number of coils of the polar filament, of Thelohania sp. was similar to that of T. solenopsae published by Knell and Allen (1977). The single Thelohania sp. free spore observed in this study had 15 coils whereas Knell and Allen (1977) report 9-11 coils. Coils of the polar filament are arranged irregularly in both Vairimorpha sp. and V. invictae (Jouvenaz and Ellis 1986) but the number of turns was between 11-13 in Vairimorpha sp. and ~ 9 in V. invictae. Free spores of Vairimorpha sp. and V. invictae are similar in structure and number of turns of the polar filament (24-26 coils in V. invictae and ~ 26 coils in Vairimorpha sp.).

Furthermore, no data are available on host specificities of T. solenopsae, V. invictae, Thelohania sp. and Vairimorpha sp. Andreadis (1994) and Garcia and Becnel (1994), in utilizing features of the polar filament as a distinguishing character to describe new species, placed great weight on the fact that the microsporidian species are all from different mosquito hosts (which do not interbreed). In other words, microsporidian species, that are ultrastructurally nearly identical except for characteristics of the polar filament, probably could not be differentiated easily from each other if they would coinfect the same host. It is not known whether Thelohania sp. and Vairimorpha sp. from S. richteri can infect S. invicta (the host of T. solenopsae and V. invictae) and vice versa. To complicate the matter further, it is not even clear whether S. richteri and S. invicta are two distinct species (Vander Meer and Lofgren 1988). Thus, the observations on differences in polar filament features did not provide a good taxonomic character to separate the fire ant microsporidia from each other because of (1) inadequate sample sizes and (2) lack of knowledge of host specificities.

Another problem that complicates comparing microsporidia at the ultrastructural level, involves sample preparation for TEM. It is a technical challenge to get good spore infiltration of the imported fire ant microsporidia for tissue fixation and embedding into plastic. Because masses of spores occur in cysts, it is nearly impossible to get good infiltration of spores towards the interior of the cyst. Most importantly, the spore walls present a nearly impenetrable barrier to fixatives and plastics (J. Becnel, personal communication). Vairimorpha sp. and V. invictae meiospores are especially challenging because of their very thick spore walls. The obtainable ultrastructure of the fire ant microsporidian spores thus does not result in the same quality of resolution available from some other species of microsporidia such as those parasitizing aquatic insects which are (for unknown reasons) easier to prepare for TEM.

In conclusion, ultrastructural evidence indicates that Thelohania sp. and T. solenopsae appear to be the same species. Ultrastructural evidence also indicates that Vairimorpha sp. and V. invicta are conspecific. It is premature, though, to draw a conclusion based solely on morphological characteristics because there are examples of microsporidia that are distinct species based on biochemical and immunological tests but indistinguishable at the light- and ultrastructural level (Didier et al. 1991). Differences in number and arrangement of coils of the polar filament presently cannot be used as good taxonomic characters because of problems of quantification and unresolved host specificities. Vairimorpha sp. and Thelohania sp. are very different from each other at the gross morphological and ultrastructural level, but because of the occurrence of heterosporous microsporidia and phenotypic plasticity more evidence is needed to resolve the question whether they are different phenotypes of the same species.

CHAPTER III

FATTY ACID METHYL ESTER ANALYSIS IN MICROSPORIDIA: EVALUATION OF A NEW TOOL FOR IDENTIFICATION

Introduction

Literature Review

This literature review will highlight several significant developments in the use of fatty acid analysis as a tool for rapid identification and taxonomy of microorganisms. A comprehensive review on gas chromatographic analyses of fatty acids in bacteria and other microorganisms, using capillary columns, was written by Welch (1991). Asselineau (1962) provided an overview of the early work on bacterial lipids performed by open, packed glass columns. O'Leary (1975) reviewed microbial lipids and their role in taxonomy, phylogeny, and identification of bacteria. Supplementary references may be obtained from a number of books on microbial chemotaxonomy and lipids (Goodfellow and O'Donnell 1994; Ratledge and Wilkinson 1988; Goodfellow and Minnikin 1985).

Lipid composition, especially fatty acid composition, has been an important criterion in determining taxonomic relationships among bacteria (Shaw 1974). Analysis of fatty acid methyl esters (FAMEs) for identification, first applied to bacteria, is now a routine practice for anaerobic and aerobic bacteria (Dees et al. 1975; Moss et al. 1974; Moss 1981; Sasser 1990a). FAME analysis has recently been applied to the taxonomy and identification of yeasts (Kock 1988; Sasser 1990c) and glomalean endomycorrhizal fungi (Jabaji-Hare 1988; Bentivenga and Morton 1994; Graham et al. 1995). A recent publication by Van der Westhuizen et al. (1994) reports on FAME profiles in Chytridiomycota, Zygomycota, and Deuteromycotina.

James and Martin (1952) first reported on the application of gas-liquid partition chromatography to the separation and micro-estimation of volatile fatty acids. This method was successfully used by Abel et al. (1963) to analyze fatty acids of eleven bacteria. They demonstrated qualitative and quantitative fatty acid profile differences among selected families in the class Schizomycetes, and quantitative differences among five selected genera of the family Enterobacteriaceae. Furthermore, they found that media components and growth stage influence bacterial fatty acid composition. Their studies established the potential usefulness of cellular fatty acid analysis in bacterial taxonomy, and laid the foundation for further investigations. For example, Yamakawa and Ueta (1964) used gas chromatography to determine fatty acid and monosaccharide compositions of whole bacterial cells of seven species of Neisseria. Other early studies were concerned with various aspects of culture conditions that influenced fatty acid composition of Escherichia coli, such as temperature and growth media (Marr and Ingraham 1962; Knivett and Cullen 1965). A wealth of literature exists on the influence of nonstandardized growth conditions on the fatty acid composition of organisms. Recent research has been undertaken to determine the effect of culture age on FAME profiles of lactic acid bacteria (Decallone et al. 1991). Effects of growth temperature on FAME profiles of Bacillus subtilis and B. megaterium (Suutari and Laakso 1992) and culture media on FAME profiles of B. anthracis and B. cereus (Lawrence et al. 1991) have been reported. In addition to bacteria, studies on FAME profiles of yeasts including several Candida species, Torulopsis glabrata, and Cryptococcus neoformans (Marumo and Aoki 1990) and Mortierella alpina (Shimizu et al. 1991) are available.

The source of fatty acids in microbial cells is lipid. Lipids are substances of biological origin that are soluble in organic solvents such as chloroform and ether but only sparingly soluble in water (Voet and Voet 1990). Fats, oils, fat-soluble vitamins such as A, D, E, and K, and some hormones are lipids. Fatty acids are carboxylic acids having hydrocarbon backbones ranging from 1-30 carbons in length. They are rarely found as free acids in the cell but, rather, occur as esters of glycerol. Some free fatty acids are toxic to living cells (Wood 1988). The fatty acids present in microbial lipids are generally of four types: straight-chain or saturated, mono- or polyunsaturated, branched-chain (predominantly iso and anteiso), and substituted fatty acids. The latter group includes cyclopropane, hydroxy, and alcohol moieties (Schweizer 1988).

Palmitic acid (16:0) is highly conserved in prokaryotes (Welch 1991). Branched-chain and cyclopropane fatty acids characterize many gram-positive and gram-negative bacteria, but are not found in eukaryotic microorganisms. Polyunsaturated fatty acids, found in higher organisms, are not biosynthesized by aerobic bacteria (Welch 1991). Hydroxy acids are typical of gram-negative, but not gram-positive bacteria. Mycolic acids are representative of the Actinomycetes. Strict anaerobes and archaebacteria synthesize plasmalogens (ether-linked lipids). An excellent summary on the distribution of fatty acids among major taxonomic groups is given by Kerwin (1994). Additional information on fatty acids characteristic of different microorganism can be found in Welch (1991) and Ratledge (1988). Fungal fatty acids are discussed by Van der Westhuizen et al. (1994) and Lösel (1988).

Several technical advances have simplified the use of cellular fatty acid analysis as a diagnostic tool while increasing accuracy and precision. Generally, gas-liquid chromatography (G-LC) of FAMEs had been done on glass columns of variable lengths with internal diameters from 2-4 mm, and packed with polar or nonpolar stationary phase material (Moss 1981). Packed columns enable larger sample volumes to be assayed, but do not separate all of the types of substituted acids biosynthesized by bacteria. For example, hydroxy acids appear as 'shoulder peaks' on the leading or tailing edge of other peaks on a chromatogram of a packed column, and cis/trans isomers of some acids with the same carbon chain length may appear as one peak or will not be resolved at the base line (Moss et al. 1980; Moss 1981). The introduction of flexible fused silica glass capillary columns with internal diameters of 0.2 mm and wall-coated, rather than packed stationary phases enables increased resolution of fatty acids. Hydroxy acids and most structural isomers appear as sharp, symmetrical, well resolved peaks when fused silica capillary columns are used (Moss et al. 1980).

Other developments include the modification of a fatty acid extraction method to obviate the use of hazardous diethylether (Moss et al. 1974; Moss 1981). An esterification protocol was modified to improve total fatty acid recovery as well as reduce hydroxy acid tailing and cyclopropane acid degradation (Miller 1982). Finally, computerized data reduction programs facilitate rapid analysis of large data sets (Aston 1977; Eerola 1988; Sasser 1990a).

A commercial, microbial identification system based on FAME profile analysis, the Microbial ID Inc. (MIDI) automated Microbial Identification System (MIS), has been developed (Sasser 1990a). MIDI has created data bases of FAME profiles for identification of aerobic and anaerobic bacteria, including actinomycetes, yeasts and other fungi (Sasser 1990b; 1990c). Stead et al. (1992) assessed the MIDI system by comparing FAME profiles of 773 strains of plant pathogenic bacteria representing 25 taxa and related saprophytic bacteria. They found that the confidence of correct identification is very high at the genus and species levels, but lower at the subspecies and pathovar levels. Jarvis and Tighe (1994) found that the MIDI system correctly identifies recognized species of Rhizobium with high accuracy.

This study assessed for the first time the application of FAME profiles for the identification of microsporidia, represented by three species of three genera. Due to sample size limitations, only Thelohania sp. of the fire ant microsporidia could be included; inadequate spore material was available of Vairimorpha sp. and T. solenopsae.

Materials and Methods

Test organisms

Three genera were selected for fatty acid analysis: Vairimorpha, Nosema, and Thelohania. Vairimorpha necatrix was obtained from J.V. Maddox, Illinois Natural History Survey, and propagated in the corn earworm, Helicoverpa zea. Nosema algerae, provided by A.H. Undeen, USDA-ARS, Gainesville, was augmented in H. zea and the common malaria mosquito, Anopheles quadrimaculatus. Thelohania sp. was harvested from field-collected Argentine fire ants, S. richteri, courtesy of R.S. Patterson and J. Briano.

Spore Propagation of N. algerae in H. zea

Four-day-old H. zea larvae were starved individually for 24 h, then 20 µL of an aqueous suspension of 1×10^7 N. algerae spores/mL was added to each. After an additional 24 h, the larvae were placed separately on a pinto bean diet, and maintained at 29°C. Spores were purified from adult H. zea.

Spore Propagation of V. necatrix in H. zea

Five-day-old H. zea larvae were exposed to 10 µL of 1×10^6 V. necatrix spores/mL each and raised separately on pinto bean diet at 29°C. Spores were harvested from last instar H. zea larvae.

Spore propagation of N. algerae in A. quadrimaculatus

Approximately 1000 mosquito eggs were hatched in 100 mL water, thereafter called infusion water, containing 13 mg of a 1:1 mix of dried, powdered liver and brewer's yeast. After 24 h, this infusion was enriched with 30 mg of alfalfa powder, and the larvae were exposed to 1×10^5 spores of N. algerae . One day post-exposure, the larvae were transferred to 8x38x50 cm rearing pans containing 900 mg powdered alfalfa in 3 L deionized water. After two days, 900 mg of a 1:1 mix of liver:brewer's yeast was added; thereafter, in 2-3 day intervals until pupation, 900 mg of a 1:1:1 mix of liver: brewer's yeast: hog chow was added. Pupae were picked daily, transferred to small cups, and held for emergence in aluminum C-frame cages covered with tube gauze. Cotton balls saturated with 10% aqueous solution of sucrose were added (Anthony et al. 1978). Spores were harvested from adult mosquitoes 3-5 days post-emergence.

Spore Harvest and Purification

Last-instar H. zea larvae infected with V. necatrix were surface-sterilized in 70% ethanol. Fat bodies were removed without lacerating gut tissues, placed into deionized water, ground in a glass tissue grinder, and filtered through cotton. Adult H. zea moths infected with N. algerae were rinsed in water and, after wing removal, triturated in a Tekmar Tissumizer in deionized water. The resulting suspensions were filtered through a cotton plug in a glass syringe. The crude V. necatrix and N. algerae spore preparations were further purified by a deionized water wash and differential centrifugation on a continuous Ludox gradient (Undeen and Alger 1971).

Adult infected mosquitoes were immobilized by chilling at -20°C for about 3 min, removed with an aspirator connected to a vacuum pump, and homogenized in a small amount of deionized water in a Waring blender. The resulting suspension was strained through a cotton plug in a syringe to remove large body parts. Further purification was achieved by a deionized water wash and differential centrifugation on a continuous Ludox gradient.

Adult worker ants of S. richteri infected with Thelohania sp. were triturated in a Tekmar Tissumizer in 'ant homogenizing buffer' (0.1% SDS, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). The suspension was strained through cotton and centrifuged in deionized water. The pellet was incubated for 10 min at 40°C in 10 µg/mL proteinase K and 1/4 vol of interfacial envelope disruption buffer (4% SDS, 25 mM EDTA, 50 mM Tris-HCl pH 7.5), followed by differential centrifugation on a Ludox gradient. All spore preparations were further cleaned by centrifugation on a 100% Percoll gradient, and repeatedly washed in deionized water, prior to fatty acid analysis.

Fatty acid extraction and analysis

Spore preparations used for fatty acid analysis were examined with a phase contrast compound microscope prior to extraction to check for bacterial contaminants. Approximately 1×10^9 spores in aqueous suspension were pipetted into a 13x100 mm glass test tube and stored overnight at 4°C to allow the spores to settle. Prior to extraction, spore samples of N. algerae from the two insect hosts were rinsed with 0.1% SDS to remove externally attached host lipids. Analysis of the SDS rinsate indicated that no fatty acids, from 9-20 carbons in length, were present. The next day, the supernatant was withdrawn carefully and esterification of fatty acids was accomplished using the method of Miller (1982). Approximately 1×10^9 spores were pipetted into a 13x100 mm culture tube and stored overnight at 4°C to allow the spores to settle. The next day, the supernatant was withdrawn carefully and 1 mL of 15% NaOH in 50% methanol was added. The tube was capped, and fatty acids were saponified at 100°C for 30 min. Upon cooling, 2 mL of 6 N HCl in 50% MeOH were added, the tube was recapped and heated at 80°C for 10 min to methylate the fatty acids. Fatty acid methyl esters (FAME) were solvent-extracted from the aqueous phase with 1.25 mL of hexane:methyl-tert-butyl ether (1:1; v/v). The organic phase was washed with 3 mL of 1.2% aqueous NaOH and transferred to a gas chromatograph (GC) vial. To determine the number of microsporidia required for fatty acid analysis, a range of different numbers of spores (3.3×10^8-2.1×10^9) was extracted.

Although fewer numbers of spores could be extracted and derivatized, a sample size of $1x10^9$ spores is recommended to provide sufficient area count.

Fatty acid methyl ester extracts were analyzed by the Microbial ID System (MIDI) (Sasser 1990b), which consists of a computer-linked Hewlett Packard 5890 gas-liquid chromatograph fitted with an Ultra 2 fused silica capillary column (25 m x 0.2 mm i.d. x 0.33 µm film thickness; crosslinked 5% phenyl methyl silicone). Following a 1/100 split injection of 2 µL at 250°C, the oven temperature was increased 5°C/min from 170°C to a final temperature of 270°C; hydrogen was used as the carrier gas. After flame-ionization, FAME peaks were quantified by a Hewlett Packard 3392 integrator and expressed as percentages of the total FAME profiles. Data were stored in the MIDI computer for subsequent comparison and statistical analyses. Prior to and between every ten-sample analyses, a calibration standard mixture consisting of the 12 straight-chain carbon acids from C9 to C20, plus five hydroxy acids, was injected. The resulting retention time and quantitative data served as quality control indicators to ensure good column performance and peak matching by the MIDI system. Periodically, Stenotrophomonas maltophilia, a bacterium whose FAME profile is well characterized, was used as a positive control to ensure reproducibility among different extraction batches.

Representative samples were further characterized by coupled GC-mass spectrometry (GC-MS). Aliquots of the microsporidian FAME mixtures and two FAME standards (MIDI Calibration Standard Mix from 9-20 C; Applied Science Division Standard with saturated and unsaturated C18) were analyzed by a Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector (ITD, Model 6210), with INCOS data collection software and a 80286 computer. The GC-MS was fitted with a 25 m x 0.25 mm i.d. DB-1 fused silica capillary column. The injection of 1 µL was in a splitless mode, followed with a purge flow of helium after 30 sec. The carrier gas was helium with a flow rate of 25 cm/sec (Nation et al. 1992). The initial temperature of the column was 60°C;

after sample injection, the temperature was programmed to 150°C at 30°C/min, then raised to 220°C at 5°C/min and held for a total running time of 100 min.

Mass spectrometry and comparison of the resulting peaks to the MIDI calibration standard mix and peak library were employed to confirm the identity of the major acids detected in the microsporidia.

Data analysis

Fatty acid methyl esters of the samples were named by comparing their retention times to those of the calibration standard (a mixture of straight-chain saturated fatty acids from 9-20 carbons in length including 5 hydroxy acids). Retention time data from the calibration mixture were converted to Equivalent Chain Length (ECL) data for fatty acid naming. Thus, the ECL value for each compound to be analyzed was computed, and the compounds were named based on comparisons to the standards as well as the ECL of acids stored in the MIDI peak library (142 peaks total) (Sasser 1990b).

A library was created from the microsporidia FAME profiles, and relationships among samples were analyzed with Principal Component Analysis. Measurements of variability and clustering among the profiles were portrayed by plotting the percentages of the three major fatty acids on a 3-D graph.

Statistical analysis of the fatty acid data was performed with an analysis of variance (ANOVA) followed by a Tukey's mean separation test to compare the means of each fatty acid among the microsporidian species tested (SAS Institute 1989).

Results

Three acids, palmitic (16:0), oleic (18:1 ϖ 9 cis), and two closely-eluting peaks denoted as Summed Feature 6 (18:2 ϖ 6,9 cis/18:0 anteiso) comprise 60% or more of the total FAME profiles of V. necatrix, N. algerae and Thelohania sp. FAME profiles were qualitatively and quantitatively distinct for the three species. Myristic acid (14:0) and 20:1 ϖ 9 cis were present at low levels in all of the *N. algerae* and *Thelohania* sp. samples. However, these acids were not detected in *V. necatrix* (Table 3.1). Quantitative differences include significantly lower levels of palmitic acid (16:0) in *Thelohania* sp. than the other two species ($p<F = 0.0001$; d.f. = 3,22; $\alpha = 0.05$) and significantly higher levels of myristic acid (14:0) and 20:1 ϖ 9 cis in *Thelohania* sp. than *N. algerae* ($p<F = 0.0001$; d.f. = 3,22; $\alpha = 0.05$). Oleic acid (18:1 ϖ 9 cis) was present at significantly higher amounts in *V. necatrix* than in *N. algerae* ($p<F = 0.0001$; d.f. = 3,22; $\alpha = 0.05$).

Three fatty acids not present in the MIDI Microbial Identification System (MIS) peak library index were found in several *Thelohania* sp. samples (Table 3.1). Similar instances of unnamed fatty acids have also been reported in recent studies on vesicular-arbuscular mycorrhizae (N.C. Hodge, personal communication). The three unnamed acids comprised 16.3 ± 9.8% of the total FAME profiles in *Thelohania* sp. samples. The acids eluted from the MIDI system's capillary column at 9.5, 11.9, and 12.5 minutes and had calculated chain lengths of 15.6, 17.0, and 17.4 carbons, respectively. Structural determination of the unnamed acids would be best confirmed using ancillary GC-mass spectrometry and nuclear magnetic resonance techniques.

Host influence on FAME profiles of the spores was tested by producing *N. algerae* in two different insects: the corn earworm, *H. zea* and mosquito, *A. quadrimaculatus*. Three closely-eluting 18:1 cis-trans isomers, combined as Summed Feature 7 (18:1 ϖ 7 cis/18:1 ϖ 9 trans/18:1 ϖ 12 trans), and arachidonic acid (20:4 ϖ 6,9,12,15 cis), were detected in *N. algerae* isolated from mosquito at 6.3% and 4.1%, respectively (Table 3.1). Neither acid, however, was present in *N. algerae* from corn earworm. Furthermore, the percentages of three other acids varied according to host.

Table 3.1. Fatty Acids (%) in Three Microsporidian Species from One Aquatic and Two Terrestrial Insect Hosts

Fatty Acid[a]	Nosema algerae				Vairimorpha necatrix		Thelohania sp.	
	A. quadrimaculatus		H. zea		H. zea		S. richteri	
	n=7		n=7		n=5		n=7	
14:0	2.8 ± 0.2[d]	B[e]	2.2 ± 0.1	C	0.0	D	4.7 ± 0.2	A
16:1$_{\varpi 7}$cis	11.4 ± 0.8	A	2.0 ± 0.1	C	1.4 ± 0.2	C	4.2 ± 0.7	B
16:0	34.5 ± 0.6	A	34.1 ± 0.5	A	31.2 ± 1.5	A	22.7 ± 1.0	B
Summed Feature 6[b]	13.4 ± 0.8	B	25.4 ± 0.8	A	28.4 ± 2.2	A	17.4 ± 1.3	B
18:1$_{\varpi 9}$cis	16.5 ± 0.3	C	20.5 ± 1.8	BC	29.6 ± 2.9	A	26.0 ± 2.1	AB
Summed Feature 7[c]	6.3 ± 0.5	A	0.0	B	0.0	B	0.2 ± 0.2	B
18:0	4.7 ± 0.2	BC	9.4 ± 0.2	A	6.5 ± 0.9	B	4.1 ± 0.6	C
20:4$_{\varpi 6,9,12,15}$cis	4.1 ± 0.3	A	0.0	B	0.0	B	2.7 ± 0.6	A
20:1$_{\varpi 9}$cis	1.8 ± 0.4	BC	2.6 ± 0.1	B	0.0	C	6.3 ± 0.8	A

[a] Number of carbon atoms in fatty acid:number of double bonds per acid

[b] Summed Feature 6 identified as 18:2$_{\varpi 6,9}$cis/18:0 anteiso by the MIDI peak library

[c] Summed Feature 7 identified as 18:1$_{\varpi 7}$cis/$_{\varpi 9}$trans/$_{\varpi 12}$trans by the MIDI peak library

[d] Standard errror of the mean

[e] Arithmetic means in a row followed by a different letter are significantly different from each other

In N. algerae from corn earworm, levels of 16:1 ϖ 7 cis (palmitoleic acid), 18:2 ϖ 6,9 cis/18:0 anteiso (Summed Feature 6), and 18:0 (stearic acid) were 2.0%, 25.4% and 9.4%, respectively. Percentages of those acids in N. algerae isolated from mosquito, 11.4%, 13.4%, and 4.7%, were significantly different ($p<F = 0.0001$; d.f. = 3,22; $\alpha = 0.05$).

Based on mass spectral information, the major FAMEs present in the microsporidia tested (14:0, 16:0, 16:1 ϖ 7 cis, 18:0, 18:1 ϖ 9 cis, 18:2 ϖ 6,9 cis) could be confirmed. Chromatograms of the FAME standards and the microsporidia are presented in Figures 3.1-3.3. The chromatograms were obtained from samples characterized by coupled GC-MS. With this system, the C20 fatty acids could not be detected.

Principal Component Analysis of the three major acids was used to graphically portray the clustering of the three species (Figure 3.4). Thelohania sp. was separated from the other species because of its low percentage of 16:0. Nosema algerae and V. necatrix both had larger percentages of 16:0, but V. necatrix also had a high percentage of 18:1 ϖ 9 cis, which distinguished it from N. algerae.

Host insect affected the clustering of N. algerae. Lower levels of Summed Feature 6 were detected in N. algerae from mosquito than N. algerae from corn earworm. Variability in the FAME profiles of the individual samples was lowest in N. algerae from mosquito (Table 3.1, Figure 3.4). Comparison of analyses of fresh and stored spore samples (storage at 4°C for 3 months, or -70°C for 1 month, of N. algerae and Thelohania sp., respectively) showed that storage did not alter the FAME profiles (data not shown).

Discussion

Fatty acid methyl ester profile analyses of microsporidia have not been reported previously. The three microsporidian species analyzed in this study can be differentiated

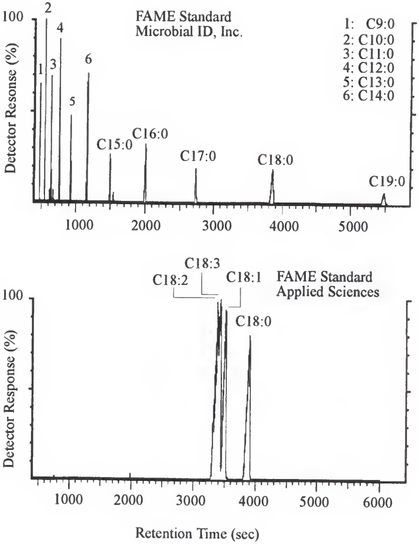


Figure 3.1. Gas chromatograms of FAME standards. Standards were analyzed with a computer-linked Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector with INCOS data collection software.

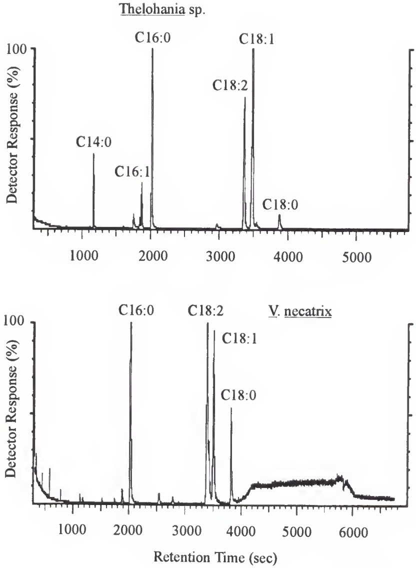


Figure 3.2. FAME chromatograms of Thelohania sp. and V. necatrix. Fatty acids were saponified, methylated, solvent-extracted, base-washed and analyzed by a computer-linked Hewlett-Packard 5890 gas-liquid chromatograph and a computer-linked Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector with INCOS data collection software.

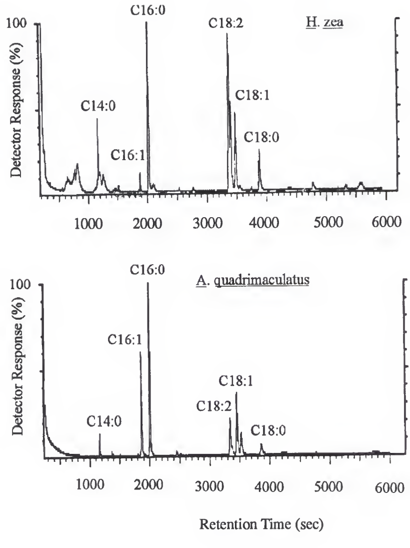


Figure 3.3. FAME chromatograms of N. algerae in two different insect hosts. Fatty acids were saponified, methylated, solvent-extracted, base-washed and analyzed by a computer-linked Hewlett-Packard 5890 gas-liquid chromatograph and a computer-linked Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector with INCOS data collection software.

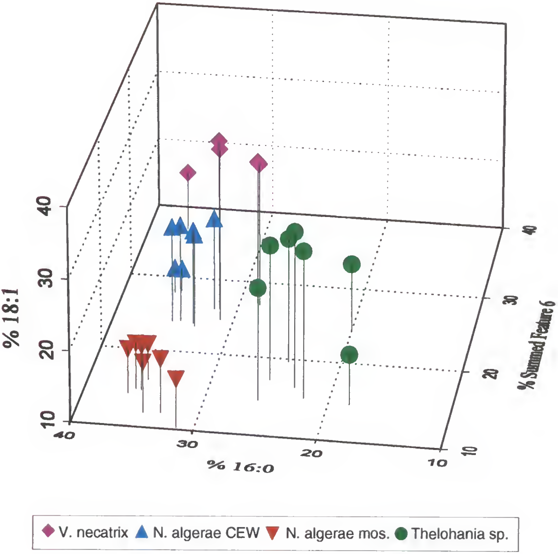


Figure 3.4. Three major fatty acids of three species of microsporidia. Measurements of variability and clustering among the profiles were portrayed by plotting the percentages of the three major fatty acids on a 3-D graph. CEW = corn earworm, mos. = mosquito.

by a combination of qualitative and quantitative FAME profile characteristics (Table 3.1, Figure 3.4). Three acids, palmitic (16:0), Summed Feature 6 (18:2 ω 6,9 cis/18:0 anteiso) and oleic (18:1ω 9 cis) were present in large percentages in all microsporidian samples analyzed. Palmitic acid is ubiquitous; it is present in all organisms (M. Sasser, personal communication). Oleic acid is present in many bacterial human pathogens, phytopathogens, as well as vesicular-arbuscular mycorrhizae. Examples include gram positive bacilli and mycobacteria (Welch 1991; Portaels et al. 1993), the plant pathogens *Erwinia amylovora*, *E. carotovora*, *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*), and *P. syringae* (Sasser 1990a), and the mycorrhiza *Gigaspora rosea* (Bentivenga and Morton 1995). A comprehensive list of fatty acids characteristic of a wide variety of organisms has been published (Kerwin 1994).

Fluctuations in FAME profiles were especially evident in *V. necatrix* and the *Thelohania* sp. For example, three unnamed fatty acids were detected in several, but not all, *Thelohania* sp. samples. These acids could be typical of developing spores (physiological age differences of spores), or representative of the organism. The microsporidian life cycle has two distinct phases: merogony (or schizogony) and sporogony. Vegetative stages called meronts (or schizonts) develop into sporonts, and finally into mature spores (Sprague et al. 1992). Preliminary experiments indicated that FAME profiles of immature spores were qualitatively and quantitatively different from profiles of mature spores of the same species (data not shown). Density gradient centrifugation of microsporidian spores may not always result in the complete separation of immature and mature spores. Spore bands with predominantly mature spores may therefore contain immature spores (and vice versa), rendering spore samples not completely homogeneous, which may add variability to the FAME profiles.

A variety of environmental factors, including age, culture medium, pH, and growth temperature have been shown to affect FAME profiles of bacteria (Lechevalier 1989; Decallonne et al. 1991; Shimizu et al. 1991; Stead et al. 1992) and fungi (Marumo and

Aoki 1990; Van der Westhuizen et al. 1994). FAME profiles of other types of organisms may also be influenced by environmental, physiological, and developmental changes; for example, diet and development strongly influence profiles of insects (Stanley-Samuelson et al. 1988). In bacteria, stability of the FA composition is achieved through growth under standardized conditions in vitro. FA stability is optimal in bacteria growing at the late log or early stationary growth phase (Sasser 1990b). Nosema algerae had a qualitatively and quantitatively distinct profile, depending on the host (Table 3.1, Figure 3.1). Host influence on FAME profiles of microsporidia may render fatty acid analysis unsuitable as a tool for microsporidian identification unless culture conditions for all microsporidia can be standardized (in vitro culture in either cell-free media or in cell lines). In vitro culture of certain microsporidian species has been accomplished (Kurtti et al. 1994, Undeen 1975).

Conversely, spores of several species of glomalean fungi yielded reproducible FAME profiles despite being grown in association with different host plants and with contaminating microorganisms present (Graham et al. 1995). Glomalean endomycorrhizal fungi are similar to many microsporidia in that they cannot be cultured without their hosts; each form obligate symbiotic relationships with the roots of many plant species. Taxonomy of glomalean fungi is currently based on spore morphology. This is similar to microsporidia where assessment of diversity using only morphological characters is difficult because of inadequately defined characters, and ambiguous distinction between morphologically similar species (Morton and Benny 1990; Morton 1993). Molecular and biochemical characters are needed to supplement the morphological data.

Host influence on the FAME profile of microsporidia is one problem that needs to be solved by standardizing culture conditions. Another problem is the requirement of a large number of spores for fatty acid extraction. The large sample size of roughly 1×10^9 spores for fatty acid extraction makes FAME analysis not practical for many microsporidia because of in vivo and in vitro culture limitations. However, Welch (1991) pointed out that the fatty acid extraction process could be scaled down to accommodate for smaller sample sizes.

More studies with additional microsporidian species from a variety of environmental conditions (e.g. different animal host) are needed to determine fatty acids characteristic for the microsporidia, and to assess qualitative and quantitative aspects of FAME analysis as a discriminant tool in identification. Microsporidian culture conditions should be standardized, and sample size requirements reduced. The compilation of microsporidian FAME profiles will enable statistical comparisons, using the MIDI pattern recognition software. By comparing profiles of well-characterized reference microsporidia, the utility of FAME analysis as a taxonomic tool for the identification of microsporidia could be evaluated.

CHAPTER IV
COMPARATIVE MOLECULAR CHARACTERIZATION OF MICROSPORIDIA FROM SOUTH AMERICAN FIRE ANTS

Introduction

Literature Review

Molecular structures and sequences generally are better indicators of evolutionary relationships than classical phenotypes. Thus, the basis for the definition of taxa has progressively shifted from the organismal to the cellular to the molecular level (Woese et al. 1990). Zuckerkandl and Pauling (1965) first discussed the role of 'informational macromolecules', molecules that carry the genetic information or an extensive translation thereof (DNA, RNA, proteins), as potentially the most informative taxonomic characters and not just one type of characters among other, equivalent types. They viewed the genetic information encoded in these molecules as documents of the evolutionary history of organisms and proposed to use these molecules in creating a molecular phylogeny.

Microorganisms frequently lack distinctive morphological, developmental, and nutritional characteristics that could be used in systematic analysis (Lane et al. 1985). It was thus a group of bacteriologists, under the direction of C. Woese, who produced the first comprehensive phylogeny of prokaryotes (indeed of life on earth) based on 16S rRNA partial sequences (Woese et al. 1977; Fox et al. 1980). Their studies revealed that instead of two major kingdoms -prokaryotes and eukaryotes-, living systems could be divided into three major evolutionary lineages: archaebacteria, eubacteria, and eukaryotes.

Each of the primary kingdoms has its particular form of rRNA. Strong 16S rRNA sequence signatures, i.e. positions in the molecule that have a highly conserved or invariant composition in one kingdom, but a different (highly conserved) composition in one or both of the others, define and distinguish the three urkingdoms (Woese 1987). Analysis of the sequence of the small subunit rRNA gene of E. coli revealed that 'universally conserved' elements (short sequences that appear to be conserved in all organisms) are distributed along the entire length of the E. coli 16S rDNA. Similar sequence analyses of small subunit RNA genes from a diverse group of organisms confirmed this observation and identified the existence of 'kingdom-specific' conserved elements (sequences that are conserved only in the eubacteria, archaebacteria, or eukaryotes, respectively) (Sogin and Gunderson 1987). There is a clear tendency for universally conserved nucleotides to fall in unpaired regions of the rDNA. (Gutell et al. 1985).

Increasingly, systematics of organisms is based on sequences, structures, and relationships of molecules, with phenotypic and biochemical properties being used to support these findings (Sogin and Gunderson 1987; Woese et al. 1990). For largely historical and practical reasons, most systematic research has focused on a small subset of genes, especially nuclear rRNA and mitochondrial rRNA and protein-encoding genes. RNA sequencing was feasible before DNA sequencing. Mitochondrial DNA occurs in multiple copies in the cell and is relatively easy to manipulate (Brower and de Salle 1994). Apart from historical and practical considerations, ribosomal genes are particularly well suited for defining evolutionary and systematic relationships because they are universally distributed and functionally homologous in all known organisms (Olsen et al. 1986; Hillis and Dixon 1991). Generally, there are three rRNAs in prokaryotes and four nuclear rRNAs in eukaryotes. The RNAs of bacteria are 5S (~ 120 nucleotides), 16S (~ 1500 nucleotides), and 23S (~ 2900 nucleotides). The nuclear RNAs of eukaryotes are 5S, 5.8S (~ 160 nucleotides), 18S (~ 1800 nucleotides), and 28S (> 4000 nucleotides) (Hillis and Dixon 1991). Mitochondria and chloroplasts code for their own rRNAs ranging from 12S to 21S in mitochondria and 5S to 23S in chloroplasts.

The larger rRNAs can be used over a wide range of phylogenetic distances, from the full span of the universal tree to distinction among species within the same genus. This is due to the fact that ribosomal sequences have both highly conserved and variable regions (Olsen and Woese 1993). Different regions of the rDNA repeat unit evolve at very different rates. The most studied rRNA is the small subunit nuclear gene, 16S/18S rRNA. It has been studied most extensively because of its size and because regions of it are among the slowest evolving sequences found throughout living organisms. The slow rate of change permits the construction of many nearly universal primers. The large subunit (23S/28S) nuclear rRNA gene is larger and shows more variation in rates of evolution of its different domains than does the small subunit. The 5.8S and 5S genes are also conserved, but the shortness of the sequence greatly restricts phylogenetic usefulness. Furthermore, the larger rRNAs provide sufficient sequence information to permit statistically significant comparisons (Olsen et al. 1986).

Typically, several hundred tandemly repeated copies of rRNA genes (rDNAs) exist in a eukaryote nuclear genome. A transcription unit consists of a linear arrangement of three genes (coding for 18S, 5.8S, and 28S rRNA) which are separated by two internal transcribed spacers. An external transcribed spacer is located upstream of the 18S gene. The transcribed spacers contain signals for processing the rRNA transcripts. Adjacent copies of the rDNA repeat units are separated by nontranscribed spacers. In prokaryotes there are one to several copies of the rRNA genes, and the genes may be organized into a single operon (in which they are usually separated by the tRNA gene), or they may be dispersed throughout the genome. The gene for the 5S rRNA is closely associated with the other rRNA genes in many prokaryotes but is found elsewhere in the nuclear genome of most eukaryotes (Hillis and Dixon 1991).

The multiple copies of nuclear rRNA genes do not evolve independently but in concert (Arnheim 1983). In other words, each copy of an rRNA array is usually very similar to the other copies within individuals and species, although differences among species accumulate rapidly in parts of the array. The differences among arrays within individuals are mostly length variation in the nontranscribed spacer. The low level of heterogeneity at about 0.1% of the nucleotide positions (Mylvaganam and Dennis 1992) among rDNA within individuals (and throughout species) indicates that the multiple copies are homogenized (concerted evolution). The number of rDNA repeats, though, is known to vary widely among individuals within species that have been studied (Hillis and Dixon 1991). Exceptions to concerted evolution have been reported. For example, in the archaebacterium Haloarcula marismortui which has two nonadjacent rRNA operons, the 16S rRNA genes within the two operons differ in about 5% of the nucleotide sequence (Mylvaganam and Dennis 1992). The number of rRNA genes varies from seven in E. coli, to between 100 and 200 in lower eukaryotes, to several hundred in higher eukaryotes. Estimates of copies of the 18S-28S gene for different organisms include, for example, 150-250 in Drosophila melanogaster, 200-280 in humans, 100-140 in Saccharomyces cerevisiae, and 450 in Xenopus laevis (Lewin 1994; Gerbi 1985).

The mitochondrial rRNA genes develop much more rapidly than the nuclear rRNA genes. The spacer regions of rDNA arrays have been used less frequently for phylogenetic studies; variation in spacer regions has been used to identify species or strains, to study hybridization, and as markers in population genetics studies (Hillis and Dixon 1991).

Microsporidia are peculiar eukaryotes that lack mitochondria, peroxisomes and a 'typical' Golgi apparatus (Canning 1988). They have ribosomes with prokaryotic properties. Ishihara and Hayashi (1968) determined that ribosomes of Nosema bombycis have a sedimentation coefficient of 70S like bacteria and blue-green algae and not of 80S like the eukaryotes. The ribosomal subunits have sedimentation coefficients of 50S and 30S (typical of prokaryotes), and not of 40S and 60S (typical of eukaryotes). The small and large ribosomal subunits in turn, as determined for Thelohania maenadis and Inodosporus sp., contain 16S and 23S RNA like prokaryotes and not 18S and 28S RNA like eukaryotes (Curgy et al. 1980).

Furthermore, as shown by Vossbrinck and Woese (1986), the microsporidium Vairimorpha necatrix does not have a 5.8S rRNA. The 5.8S rRNA is a nearly universal eukaryotic characteristic. It has no size counterpart among prokaryotes although its sequence is homologous with the first 150 or so 5' nucleotides of the prokaryotic 23S rRNA. As in prokaryotes, V. necatrix has a large subunit rRNA (23S) whose 5' region corresponds to the 5.8S rRNA. Because of the unusual molecular and cytological characteristics of microsporidia, Vossbrinck et al. (1987) sequenced the 16S rRNA of V. necatrix to clarify the phylogenetic position of microsporidia. The V. necatrix 16S rRNA sequence is far shorter than a typical eukaryotic (18 S) small subunit rRNA and, at only 1,244 nucleotides, even appreciably shorter than its prokaryotic (16S) counterpart (E. coli small subunit rRNA is about 1,500 nucleotides long). They found little overall homology between V. necatrix 16S rRNA sequence and those of other eukaryotes and concluded that the lineage leading to microsporidia branches very early from that leading to other eukaryotes. It is hypothesized that some of the organisms unique features may signify a split from other eukaryotes very early in time. Kawakami et al. (1992) made yet another unusual observation: Analysis of primary and secondary structure of the 5S rRNA and rDNA of N. bombycis reveals a typical eukaryotic structure.

The objective of this study was to evaluate the taxonomic relationship of Vairimorpha sp., Thelohania sp., and T. solenopsae to each other based on their 16S rRNA gene sequences. The 16S rRNA genes (nuclear) of T. solenopsae, Thelohania sp., and Vairimorpha sp. were amplified by PCR, analyzed with restriction fragment length polymorphism (RFLP), and sequenced to gain information on the characteristics of these genes. The molecular data were used as information to evaluate the taxonomic position of the species studied. In addition, the 16S rRNA gene of Agmasoma penaei (Overstreet

1973) was amplified and sequenced. We wanted to have a 16S rRNA gene sequence of another Thelohania species to compare to the fire ant Thelohania species. The type species of the genus Thelohania is T. giardi which is found in decapods. It was described at the end of the last century (Sprague et al. 1992), and no further studies have been done with it since. Neither samples of T. giardi nor any 16S rRNA gene sequences of other Thelohania species are available to compare to those of the fire ant Thelohania species. Assuming that A. penaei, formerly called T. penaei, is closely related to the type species of the genus Thelohania, T. giardi, we chose A. penaei as a species to compare to the two Thelohania species. Both species have octosporous sporulating sequences and infect shrimp. Agmasoma penaei was moved from the genus Thelohania to its own genus, Agmasoma, because its polar filament is anisofilar (Hazard and Oldacre 1975).

Materials and Methods

Collection of Test Organisms

Thelohania sp., Vairimorpha sp., and T. solenopsae were collected from S. richteri and S. invicta respectively (chapter II). Thelohania penaei, now named A. penaei, was collected and stored in water at 4°C by R. M. Overstreet from an overwintering white shrimp, Penaeus setiferus, in a laboratory mud-substratum pond in Ocean Spring, Mississippi, on 4-12-1991.

Spore Harvest and Purification

Vairimorpha sp. spores were purified from S. richteri adults that died during the trip from Buenos Aires to Gainesville immediately upon arrival at Gainesville. The ants were ground in homogenizing buffer (10 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetate (EDTA), 0.1% SDS) in a Tekmar tissuemizer and filtered through cotton to remove large body parts. The resulting crude spore suspension was further purified by differential centrifugation on a continuous Ludox (DuPont) gradient (Undeen and Alger 1971). The spores were stored at 4°C in Tris-EDTA (TE), pH 8.0 or distilled water until DNA was extracted.

To collect Thelohania sp. spores from S. richteri and T. solenopsae spores from S. invicta, fat body cysts were dissected out of the abdomens of 25-30 infected adult workers which had been frozen at -70°C, and collected on ice in 0.1% SDS in 1.5 mL microfuge tubes. The spores were washed twice by centrifugation in deionized water, counted with a hemocytometer and stored in deionized water at 4°C until DNA was extracted. The infection level with V. invicta was so low that spores could not be purified and used in the analysis. Samples of T. penaei were obtained as an aqueous suspension from R. M. Overstreet.

DNA Extraction From Microsporidia

A DNA extraction procedure suggested by M. D. Baker and C. R. Vossbrinck (University of Illinois, Urbana, IL; personal communication) was employed. A range of spores ($1x10^5$ - $1x10^8$) was pelleted in 0.5 mL microfuge tubes by centrifugation at 10,000 g for 1 min and resuspended in 200 µL of sodium chloride/Tris/EDTA (STE) buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Approximately 200 µL 0.1 mm diameter siliconized glass beads was added to the spore suspension, and the mix was shaken in a mini beadbeater (Biospec) at low speed for 20 sec to break the spores and release their genomic DNA. Immediately after breaking the spores, the homogenate was heated at 95°C for 5 min to inactivate DNA degrading enzymes and centrifuged for 5 min at 10,000 g. The supernatant was removed, frozen solid, thawed and centrifuged again at 10,000 g for 5 min. The supernatant containing the DNA was used for PCR immediately or stored at -20°C for later PCR analysis.

PCR Of Microsporidian DNA

The 16S rRNA gene segment was amplified from the microsporidia genomic DNA by PCR using primers designed from the 5' and 3' regions. The DNA sequences for the forward and reverse primers were kindly provided by C. R Vossbrinck and M. D. Baker (personal communication). Restriction sites were incorporated into the sequences at the 5' ends to allow subsequent cloning of the PCR product. The forward primer 18f had a different restriction site sequence incorporated at the 5' end depending on whether Thelohania sp., T. solenopsae, and A. penaei or Vairimorpha sp. DNA was amplified. The forward primer JM27/18f (5'-TTTGAATTCCACCAGGTTGATTCTGCC-3') was designed to contain an *Eco*RI site (GAATTC). Another forward primer, RP6/18f (5'-AAGGTACCAGGTTGATTCTGCCTGACG-3') was designed to contain a *Kpn*I site (GGTACC). JM27/18f was used for Thelohania sp., T. solenopsae, and A. penaei DNA amplification. RP6/18f was used to amplify Vairimorpha sp. DNA. The reverse primer 1492r (5'-TTTGGATCCGGTTACCTTGTTACGACTT-3') was the same for all amplifications, and it was designed to contain a *Bam*HI site (GGATCC). Primers 18f and 1492r can be used to amplify the 16S rRNA gene of most microsporidia. Primer 18f (5'-CACCAGGTTGATTCTGCC-3') is located on nucleotides 1-18, primer 1492r (5'-GGTTACCTTGTTACGACTT-3') on nucleotides 1117-1098 on the V. necatrix 16S rDNA sequence. The sequence 5'-CAGGTTGATTCTGCC-3' of the 18f primer mismatches in two positions with a homologous sequence of primer 18e (5'-CTGGTTGATCCTGCCAGT-3') that can be used to amplify the 18S rRNA gene of many eukaryotes (Sogin and Gunderson 1987; Hillis and Dixon 1991) and prokaryotes (Elwood et al. 1985). The sequence 5'-GGTTACCTTGTTACGACTT-3' of primer 1492r is 100% homologous to Escherichia coli 16S rDNA.

PCR amplification was optimized for each new DNA template by testing 1, 5, and 10 µL (10 µL had less than 10 ng of DNA; determined by gel electrophoresis) of the crude DNA preparation with two $MgCl_2$ concentrations (1.5 mM and 2.5 mM) and two primer concentrations (4 and 8 µM) in 25 µL reaction volumes. Based on the optimizations, standard conditions for PCR were as follows: Each 50 µL reaction contained 1 µL of microsporidia genomic DNA (<< 10 ng), 4 µM of each primer (forward and reverse), 0.2 mM of each dNTP (Boehringer Mannheim), DNA polymerase and the appropriate buffer. Either 1.6 U of *Taq* DNA polymerase (Boehringer Mannheim) or 0.6 U of Primezyme™ DNA polymerase (Biometra) were used. The *Taq* DNA polymerase 1x reaction buffer contained 10 mM Tris-HCl, 50 mM KCl, and 2.5 mM $MgCl_2$. The Primezyme™ DNA polymerase 1x reaction buffer contained 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, and 2.5 mM $MgCl_2$. The reactions were overlaid with either 100 µL sterile glycerol or 50 µL Chill-out 14™ Liquid Wax (MJ Research). The reactions were carried out in an MJ Research thermocycler using the temperature profile: 94°C for 5 min, then 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 35 cycles. A final extension step of 72°C for 15 min was done after 35 cycles. A 5 µL aliquot from each reaction together with 5 µL of 1x loading dye (5% glycerol, 5 mM EDTA, 0.05% bromphenol blue) was electrophoresed on a 0.8% Seakem LE agarose gel in Tris-acetate buffer (TAE; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Ethidium bromide (EtBr) at a concentration of 0.25 µg/mL was incorporated into the gel and electrophoresis buffer to stain and visualize the DNA by UV transillumination. PCR products from three reactions were pooled and purified with the QIAquick PCR Purification Kit (QIAGEN) by following the manufacturer's instructions, eluted in sterile, distilled water or TE pH 8.0 (10 mM Tris, 4 mM EDTA) and stored at -20°C. For cloning and restriction digests, elution in TE buffer, pH 8.0, was suitable. The DNA concentration of the purified PCR product was determined by electrophoresing an aliquot with a standard of lambda (λ) bacteriophage DNA cut with *Hind*III (λ/*Hind*III cut DNA) and comparing the intensity of the ethidium bromide stained bands to each other.

Cloning of the 16S rRNA Gene

PCR products of Thelohania sp., Vairimorpha sp. and T. solenopsae were cloned into the plasmid pTZ 19R vector (Pharmacia) by transforming E. coli JM109 cells using the following protocol.

Pretreatment of PCR Product: The PCR products were digested with Proteinase K (50 µg/mL) in 10 mM Tris-HCl, 5 mM EDTA, and 0.5% SDS to remove the *Taq* DNA polymerase bound to the DNA (Crowe et al. 1991). The Proteinase K digestion was carried out at 37°C for 30 min and then treated at 80°C for 10 min to heat inactivate the enzyme. The Proteinase K treated PCR product was cleaned using the QIAquick PCR Purification Kit (QIAGEN). To check the recovery rate, a 2 µL aliquot was fractionated on a gel and the concentration estimated by comparing it with a known amount of λ/*Hind*III cut DNA as described earlier.

Restriction Enzyme Digest: Thelohania sp. and T. solenopsae PCR products were double digested with *Eco*RI and *Bam*HI (New England Biolabs) to create sticky ends for cloning. For the digest, 48 µL of amplified DNA (or 2-3 µg), 1 µL of each enzyme (20 U/µL), and 5.5 µL of the appropriate 10x restriction buffer (manufacturer's instructions) were mixed and incubated at 37°C for 4 h. Simultaneously, the plasmid DNA, pTZ 19R (1 µg/10 µL) was double digested to create compatible sticky ends. Vairimorpha sp. PCR products were digested sequentially with *Kpn*I and *Bam*HI (New England Biolabs) to create sticky ends using the appropriate buffers according to the manufacturer's instructions. For the restriction digest, 48 µL of amplified DNA, 1 µL of *Kpn*I (15 U/µL), 0.5 µL bovine serum albumine (BSA), and 5 µL of 10x restriction buffer were incubated at 37°C for 2 h. The enzyme was heat-inactivated at 70°C for 10 min, followed by the addition of 1 µL of *Bam*HI (20 U/µL), 5 µL of 10x restriction buffer and incubation for another 2 h at 37°C. Plasmid pTZ 19R DNA was digested with *Kpn*I and *Bam*HI at the same time to create compatible sticky ends. Digested PCR products were purified using the QIAquick PCR Purification Kit as described earlier and eluted in 50 µL sterile, distilled water.

The plasmid DNA was purified from SeaPlaque agarose. The digested plasmid DNA was loaded onto a 0.8% SeaPlaque agarose gel and electrophoresed to separate the 3 kb plasmid fragment from the 1.3 kb insert. The fragment was cut out, melted at 68°C for 30 min and diluted with agarose diluent (200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA) to at least 0.3% agarose concentration (Maruniak et al. 1984). The DNA was then phenol and ether extracted (3x each) and ethanol precipitated (1/2 vol of 7.5 M ammonium acetate and 2 vol of 100% EtOH, incubation on ice for 10 min, centrifugation at 10,000 g for 10 min, wash with 70% EtOH, vacuum dry). The resulting pellet was dissolved in 20 µL of distilled water.

Ligation: A 1:3 ratio in moles of vector:PCR product DNA was used (Bethesda Research Laboratories 1979). Specifically, 200 ng of pTZ 19R DNA and 250 ng of PCR product DNA were ligated to each other in a 40 µL reaction with 1 µL T4 DNA ligase (1 U/µL) and 4 µL 10x reaction buffer (New England Biolabs) at room temperature overnight in the dark (modified from manufacturer's protocol).

Transformation of E. coli JM109 Competent Cells: To inactivate the T4 DNA ligase and enhance transformation, the ligation mix was heat-treated at 65°C for 10 min. A 50 µL aliquot of E. coli JM109 competent cells was thawed on ice, and 1 or 5 µL aliquots of ligated DNA were gently mixed with the cells. The cells were sequentially incubated on ice for 30 min, heat shocked at 37°C for 30 sec, and cooled on ice for 2 min. Then, 0.95 mL of room-temperature superoptimal catabolite (S.O.C.; BRL personal communication) media was added, and the cells were grown at 37°C and 225 rpm for 1 h. A 100 µL aliquot was plated on Luria-Bertani (LB) agar media supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 20 µg/mL) and ampicillin (100 µg/mL) and incubated for 16 h at 37°C. Ampicillin-resistant clear colonies were selected as potential clones and picked off the plates for further analysis (Bethesda Research Laboratories Life Technologies, Inc. transformation protocol).

Dot Blot to Confirm Clones: All clear colonies that grew on the X-Gal and ampicillin enriched LB plate were streaked in a grid pattern on a fresh LB plate supplemented with 100 µg/mL ampicillin and incubated over night at 37°C for 16 h to develop E. coli transformant colonies. For hybridization, a nylon membrane (Zeta Probe) was cut (10 cm x 15 cm) and dots, 1 cm apart from each other, were marked on it with a soft pencil. A tooth-pick head full of each numbered E. coli transformant colony was suspended in 10 µL LB broth and 1 µL of that suspension spotted on a marked area. To denature the bacterial DNA, the membrane was sequentially immersed in 0.5 M NaOH/ 1.5 M NaCl for 30 sec, 0.5 M Tris-HCl pH 8.0/1.5 M NaCl for 5 min, and 6x standard saline citrate (SSC) for 5 min. The membrane was wrapped in Whatman Blot paper and baked at 80°C for 2 h. Prehybridization, hybridization, and washes were done in a Mini Hybridization Oven OV3 (Biometra). The membrane was prehybridized for 4-6 h at 68°C in 6x SSC, 0.5% SDS, 5x Denhardt's solution, 0.01 M EDTA, and 100 µg/mL denatured herring sperm DNA (Sambrook et al. 1989). To make the probe, 1 µg PCR product was labelled with ^{32}P-dATP using a nick translation kit (USB Nick Translation Protocol). The membrane was hybridized for 16 h at 68°C in hybridization buffer (6x SSC, 0.5% SDS, 5x Denhardt's solution, 0.01 M EDTA) containing the nick translated PCR-amplified 16S rRNA gene of the microsporidian species to be tested. The membrane was washed twice in 2x SSC and 0.5% SDS at 68°C for 2 h each and heat-sealed in a plastic bag. It was exposed to an x-ray film overnight at -70°C.

Glycerol Stock from Positive Clones: The clones that showed strong hybridization to the probe (positive clones) were picked from the LB plate and grown in 3 mL terrific broth (TB) containing 35 µg/mL ampicillin overnight at 37°C and 225 rpm. The next day, 850 µL of the cell suspension was added to 150 µL of sterile glycerol to make a 15% glycerol stock and stored at -70°C. To grow up glycerol stocks for plasmid purification, 10 µL of glycerol stock was added to 3 mL TB containing 35 µg/mL ampicillin and grown overnight at 37°C and 225 rpm.

Plasmid DNA Purification: The alkaline lysis method combined with DNA precipitation by polyethylene glycol (PEG) was used to purify the plasmid DNA carrying the cloned DNA from E. coli transformants (Nicoletti and Condorelli 1993). E. coli transformant cells were suspended in 200 µL glucose/Tris/EDTA (GTE) buffer (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) and lysed with 300 µL of 0.2 N NaOH/1% SDS for 5 min on ice. Chromosomal DNA was precipitated with 300 µL of 3.0 M potassium acetate (KOAc), pH 4.8 for 5 min on ice. After centrifugation, the supernatant was collected and treated with RNAse A (20 µg/mL) for 20 min at 37°C. After two chloroform extractions to remove proteins and residual chromosomal DNA, the plasmid DNA was ethanol and PEG precipitated (PEG precipitation: dissolve DNA pellet in 32 µL distilled H_2O, add 8 µL 4 M NaCl and mix, add 40 µL 13% PEG_{8000}, incubate on ice for 1 h, centrifuge at 4°C and 10,000 g for 15 min.) The resulting DNA pellet was resuspended in sterile distilled water. To confirm the presence of the 16S rRNA gene insert, the hybrid plasmid was digested with the appropriate restriction enzymes, and the size of the insert was compared to the purified PCR product and linearized PTZ 19R plasmid DNA by separating on a gel.

Restriction Fragment Length Polymorphisms of the 16S rDNA

Several enzymes were tested on Thelohania sp., T. solenopsae, and Vairimorpha sp.: *Sau*3A (4U/µL), *Hha*I (20U/µL), *Hae*III (8U/µL), *Aci*I (5U/µL), and a double-digest of *Hinc*II (8U/µL) and *Hind*III (20U/µL) (New England Biolabs). Digests were performed in 20 µL volumes using 12 µL of PCR product (~ 700 ng), 0.5 µL of each enzyme, 2 µL of the specific 10x restriction buffer (manufacturer's instructions), and 5.5 µL of deionized H_2O. The reaction mixes were incubated at 37°C for 2 h. The restricted DNA samples were electrophoresed with 2 µL of 10x loading dye (50% glycerol, 50 mM EDTA, 0.5% bromphenol blue) on a 3% Nusieve GTG/1% Seakem LE agarose gel.

Sequencing of the 16S rDNA

Purified PCR products of Thelohania sp. Vairimorpha sp., and A. penaei, eluted in sterile, distilled water, were used as sequencing templates. The sequence of the PCR products was completed by redundant sequencing of both strands. Hybrid plasmid DNA, carrying the cloned T. solenopsae rDNA, in sterile, distilled water was used for sequencing. The consensus sequence was obtained by redundant sequencing of both strands of three clones.

Sequencing was completed by using three primers in each direction. The sequences of the primers are listed in Table 4.1. Sequences for RP7/530f, RP9/1061f, RP8/1047r, and RP10/530r primers were obtained from C. R. Vossbrinck and M. D. Baker (personal communication).

The following primers were used for Thelohania sp.: JM27/18f, RP7/530f, RP9/1061f, RP4/1492r, RP8/1047r, and RP10/530r. The primers used to sequence T. solenopsae were M13f, RP7/530f, RP12f, M13r, RP8/1047r, and RP10/530r. Primers used to sequence the Vairimorpha 16S rRNA gene were identical to the ones used for Thelohania sp., except that RP6/18f was used instead of JM27/18f. Primer RP9/1061f was replaced with RP11f to sequence the A. penaei 16S rRNA gene, and all the other primers were the same as in Thelohania sp.

Both manual and automated DNA sequencing methods were employed. Manual cycle sequencing was performed by the dideoxynucleotide chain termination sequencing method (Sanger et al. 1977) using the fmol™ sequencing kit (Promega). For each set of sequencing reactions, 2 µL of each d/ddNTP mix (either d/ddATP, d/ddTTP, d/ddCTP or d/ddGTP) were pipetted into a 0.5 mL microfuge tubes. Then 500 fmol of template DNA (either plasmid template or PCR product), 4 pmol of primer, 6 µCi [α-^{35}S]dATP, 5 µL of fmol^{R} 5x sequencing buffer (250 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$), and sterile distilled water were combined to a final volume of 16 µL, and 1 µL of sequencing grade *Taq* DNA polymerase (5U/µL) was added to the primer/template mix. Four µL of the enzyme/primer/template mix were added to each d/ddNTP mix. The reactions were overlaid with 20 µL of Chill-out 14™ Liquid Wax (MJ Research), placed in a MJ thermal cycler preheated to 94°C, and subjected to the following temperature profile: 94°C for 2 min, then 94°C for 30 sec, 42°C for 30 sec, 70°C for 1 min (30 cycles). They were

Table 4.1. List of Sequencing Primers Used

Forward Primer	**Nucleotide Sequence of Primer (5'-3')**
JM27/18f	TTT GAA TTC CAC CAG GTT GAT TCT GCC
RP6/18f	AAG GTA CAA GGT TGA TTC TGC CTG ACG
RP7/530f	GTG CCA GC(AC) GCC GCG G
RP9/1061f	GGT GGT GCA TGG CCG
RP11f	GGT CGT TGT AAA CTC
RP12f	GGA GTG GAT TAT ACG G
M13f (-20)	GTA AAA CGA CGG CCA GT
Reverse Primer	
RP4/1492r	TTT GGA TCC GGT TAC CTT GTT ACG ACT T
RP8/1047r	AAC GGC CAT GCA CCA C
RP10/530r	CCG CGG C(GT)G CTG GCA C
M13r (-24)	AAC AGC TAT GAC CAT G

cooled to 4°C until the sequencing reactions were stopped by addition of 3 µL of fmol^R sequencing stop solution (10 mM NaOH, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanole) to each tube.

Immediately before loading the reactions on a sequencing gel, they were heated at 70°C for 2 min. The products (3.5 µL per lane) were run on a 8%, 19:1 acrylamide: bisacrylamide gel at 1800 V. After fixing (30 min in a solution of 5% acetic acid and 15% EtOH) and drying in a gel dryer on Whatman 3MM paper (1 h at 80°C), the gel was exposed to Kodak diagnostic x-ray film at -70°C (United States Biochemical Sequencing Support Service; DNA Sequencing Guide).

Automated sequencing was done by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished by employing the Taq DyeDeoxy Terminator (part number 401388) Cycle Sequencing protocol developed by Applied Biosystems (a division of Perkin-Elmer Corp., Foster City, CA) using fluorescent-labeled dideoxynucleotides. The labeled extension products were analyzed on an Applied Biosystems Model 373A DNA Sequencer.

Sequence Data Analysis

Analysis of the 16S rRNA gene sequences was done using the Genetics Computer Group (GCG) Sequence Analysis Software Package (Devereux et al. 1987) and Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 (Swofford 1993). To confirm the RFLP digests, enzyme restriction maps with the enzymes tested in the RFLP digests were created for each of the three fire ant microsporidia with MAP.

Ribosomal gene sequences of microsporidia from a variety of host organisms including insects (Hymenoptera, Lepidoptera), fish and humans and the protozooan Giardia lamblia, used as outgroup, were obtained from GenBank (G. lamblia, Sogin et al.

1989; Ameson michaelis, Zhu et al. 1993; Endoreticulatus schubergi, Ichthyosporidium sp., Nosema bombycis, Encephalitozoon hellem Baker et al. 1995; Pleistophora sp., Glugea atherinae, N. corneum, DaSilva et al. unpublished, direct submission 1994; Encephalitozoon cuniculi, Zhu et al. 1993; Enterocytozoon bieneusi, Zhu et al. 1993; Septata intestinalis, Visvesvara et al. 1995; N. apis, Malone et al. 1994; N. trichoplusiae, Pieniazek et al. unpublished, direct submission 1994; N. vespula, Ninham unpublished, direct submission 1994; V. necatrix, Vossbrinck et al. 1987). Giardia lamblia was chosen as an outgroup because, like microsporidia, it is a primitive eukaryote with a 16S like rRNA. It has two nuclei and lacks mitochondria, a normal endoplasmatic reticulum or Golgi. In a multikingdom tree based on rDNA sequences, G. lamblia represents the earliest diverging lineage in the eukaryotic line of descent. Its branching is followed by V. necatrix (Sogin et al. 1989).

A multiple sequence alignment of those sequences together with the sequences of Vairimorpha sp., Thelohania sp., T. solenopsae, and A. penaei was performed with the programs PileUp and Clustal (Genetics Computer Group, Inc., Madison, Wisconsin). The multiple sequence alignment file was analyzed with PAUP. A distance matrix was created, and the heuristic option of PAUP was used to find the optimal phylogenetic tree. A phylogenetic tree illustrates the evolutionary relationships among a group of organisms (Li and Graur 1991). A bootstrap analysis was performed to place confidence estimates on the groups contained in the optimal tree.

A distance matrix shows a pairwise comparison of all the taxa. Absolute distances could also be called absolute differences and are shown in the lower triangle of the table. Absolute distances give the numbers of nucleotides that differ between two sequences. However, a change from one state (i.e. nucleotide) to another at a particular position is counted only if that position is not missing for either of the taxa. Mean distances (given in upper triangle) are calculated by dividing the absolute distance by the total number of characters that are not missing for either taxon and thus represent the percent nucleotide difference between two taxa. Mean distances are more meaningful when some taxa have much higher proportions of missing data than others (PAUP 3.1 User's manual).

The phylogenetic tree was constructed based on the principle of maximum parsimony or minimal evolution. Maximum parsimony involves the identification of a tree that requires the smallest numbers of evolutionary changes to explain the differences observed among the taxa under study (Li and Graur 1991). In molecular phylogeny the maximum parsimony method should be called a character-state method (Li and Graur 1991) because character states are used and the shortest pathway leading to these character states is chosen as the phylogenetic tree. The heuristic option is a search using a heuristic or approximate algorithm. It was chosen because the microsporidian data set was fairly large (20 taxa) and the heuristic search generally provides the fastest way to find optimal trees.

Results

Spore Harvest

Dissection of 25-30 infected adult ants yielded about $1\text{x}10^7$-$1\text{x}10^8$ spores which amounts to approximately $3\text{x}10^5$-$3\text{x}10^6$ spores per ant. When cysts were collected in deionized water they stuck to the side of the plastic tubes which the addition of SDS prevented.

PCR of Microsporidian DNA

It was found, that with each template tested, all three DNA concentrations (1, 5, and 10 µL of crude DNA preparation with a concentration of less than 10 ng in 10 µL) were amplified and that the 4 µM primer concentration worked as well as the 8 µM primer concentration. The $MgCl_2$ concentration was the crucial factor; 2.5 mM $MgCl_2$ gave more consistent amplification results than 1.5 mM.

For DNA extraction and PCR, $1x10^7$-$1x10^8$ spores were sufficient. If DNA extraction and PCR were performed with $1x10^5$ or $1x10^6$ spores (using the same procedures as for the larger spore samples), no PCR product was obtained. A size difference existed between the amplified DNA fragments from Thelohania sp. and T. solenopsae (~ 1400 bp) to Vairimorpha sp. and A. penaei (~ 1300 bp) (Figure 4.1).

Cloning of the 16S rRNA Gene

Figure 4.2 presents a sketch on how the PCR products of T. solenopsae and Thelohania sp. were cloned into the pTZ 19R plasmid DNA. The cloned construct of Vairimorpha sp. was similar except that *Kpn*I was used instead of *Eco*RI to create sticky ends of the plasmid and PCR product DNA. The cloning procedure did not result in any clones if the PCR products were not pretreated with Proteinase K (results not shown). Proteinase K treatment was necessary to improve cloning efficiency.

Restriction Fragment Length Polymorphism of the 16S rDNA

Figure 4.3 shows three restriction cuts (*Sau*3A, *Hha*I and *Hae*III) for Thelohania sp., T. solenopsae, and Vairimorpha sp. The restriction patterns for each enzyme showed differences among Vairimorpha sp. and the two Thelohania species, but the latter two species had identical restriction profiles. As detected by gel electrophoresis, the two Thelohania species had two restriction sites each for *Sau*3A and *Hha*I, and four restriction sites for *Hae*III. The fragment sizes were roughly 750, 500, and 200 bp when cut with *Sau*3A; 760, 350, and 300 bp when cut with *Hha*I; and 750, 420, 180, 60, and 50 bp when cut with *Hae*III. Vairimorpha sp. had one restriction site for *Sau*3A (fragments

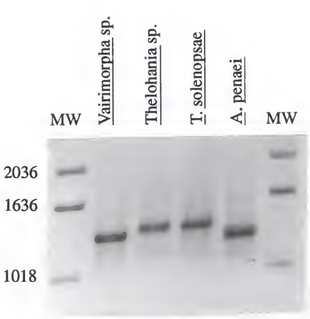


Figure 4.1. PCR products of the 16S rRNA gene of four microsporidian species. Photograph of crude PCR products following gel electrophoresis. PCR was carried out as described in the 'Methods' section. Approximately 300 ng (~ 30 ng/ µL) of each PCR product was electrophoresed with 1 µL of 10x loading dye (50% glycerol, 50 mM EDTA, 0.5% bromphenol blue) on a 0.8% Seakem LE agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). A standards, λ/*Hind*III cut DNA (200 ng) was included to determine the molecular sizes of the PCR products.

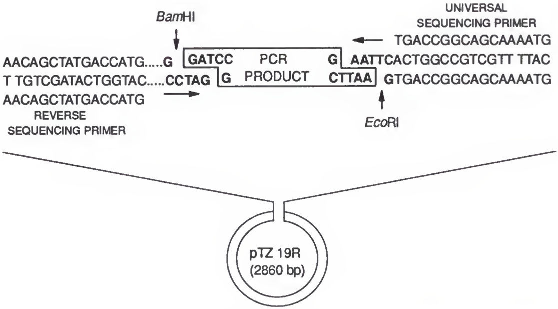
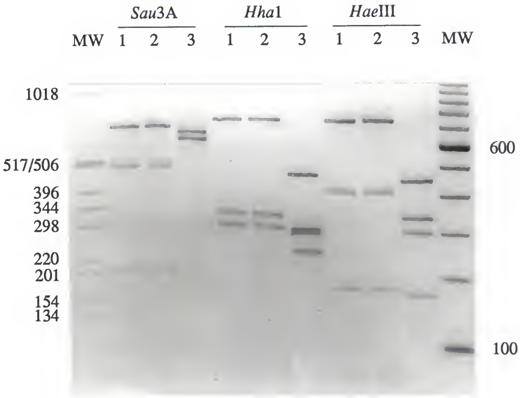


Figure 4.2. Cloned pTZ 19R Construct. The cloning procedure is described in detail in the 'Methods' section and in the appendix.

of approximately 700 and 650 bp), three restriction sites for *Hha*I (fragments of approximately 470, 300, 280, and 250 bp) and four restriction sites for *Hae*III (fragments of about 460, 350, 300, and 170, and 30 bp).

Of the other enzymes tested (results not shown), the double digest with *Hinc*II and *Hind*III separated Vairimorpha from the two Thelohania species which in turn had identical profiles. *Aci*I cut the species tested into many small fragments resulting in smears. Analysis of the completed sequences with MAP revealed that the Thelohania species each had nine restriction sites for *Aci*I. Vairimorpha sp. had twelve restriction sites for *Aci*I. The MAP analysis also showed that it did not have *Hinc*II restriction sites as opposed to the Thelohania species.



1: Thelohania sp. 2: T. solenopsae 3: Vairimorpha sp.

Figure 4.3. Restriction profiles of 16S rRNA gene PCR products of three microsporidian species. Photograph of restricted PCR products following gel electrophoresis. About 700 ng of crude PCR product of each species was digested for 2 h at 37°C with either *Sau*3A, *Hha*I, or *Hae*III in a 20 μL reaction volume. The samples were electrophoresed with 2 μL of 10x loading dye (50% glycerol, 50 mM EDTA, 0.5% bromphenol blue) on a 3% NuSieve GTG/1% Seakem LE agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Two standards, λ/*Hind*III cut DNA (200 ng) and a 1 kb DNA marker (200 ng), were included as molecular weight markers. 1 = Thelohania sp., 2 = T. solenopsae, 3 = Vairimorpha sp.

Sequencing of the 16S rDNA

The PCR primers 18f and 1492r were not suitable as sequencing primers for cycle sequencing with the fmol^{TM} sequencing kit because of problems with the sequencing reactions. Mike Baker (University of Illinois, Urbana, Il, personal communication) also was unable to use the PCR primers for cycle sequencing. He designed slightly modified sequencing primers which were moved several bases into the sequence of the PCR products. We did not design new primers because the PCR primers 18f and 1492r could be used as sequencing primers for automated sequencing by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR). Differences in experimental procedures likely account for this observation.

The 5' nucleotide of RP9/1061f mismatched with the 16S rDNA sequence of Thelohania sp. as determined by (1) failed sequencing reactions and (2) subsequent sequence comparison with the complementary strand of Thelohania sp. It did work to sequence the T. solenopsae (even though it also mismatched at the same position) but not the Thelohania sp. 16S rRNA gene in which it was replaced with RP12f.

The cloned PCR product DNA of T. solenopsae was used as sequencing template, and the sequence of the entire PCR product (1,382 bp) was obtained since sequencing primers located adjacent to the multiple cloning site were employed. PCR products of Thelohania sp., Vairimorpha sp., and A. penaei were sequenced directly. The sequenced fragments represented the majority of the PCR products (except the extreme 5' and 3' ends). The sizes of the sequenced fragments were 1,130 bp (Thelohania sp.), 1,252 bp (Vairimorpha sp.), and 1,260 bp (A. penaei). A multiple alignment of the sequences together with ribosomal gene sequences from other microsporidia is presented in Figure 4.4. Regions conserved throughout all the taxa aligned are identified by '*'.

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                   1                                              60
Thelohania sp.         ------------------------------------------------------------
T. solenopsae          ------------------------------------------------------------
N. bombycis            ----------CACCAGGTTGATTCTGCCTGACGTAG-ACGCTATACTCTAAGATTAACCC
N. trichoplusia        ----------CACCAGGTTGATTCTGCCTGACGTAG-ACGCTATACTCTAAGATTAACCC
V. necatrix            --------------------ATTCTGCCTGACGTAG-ACGCTATTCCCTAAGATTAACCC
N. vespulae            ----------CACCAGGTTGATTCTGCCTGACGTAG-ACGCTATTCCCTAAGATTAACCC
N. apis                -----------------------------GACGTAG-ACGCTATTCCCTAAGATTGGCCC
E. hellem              --------------------ATTCTGCCTGACGTGG-ATGCTATTCTCTGGGGCTAAGCC
S. intestinalis        ----------CACCAGGTTGATTCTGCCTGACGTGG-ATGCTATTCTCTGGGACTAAGCC
E. cuniculi            ----------CACCAGGTTGATTCTGCCTGACGTGG-AGGCTATTCTCTGGGGCTAAGCC
Pleistophora sp.       ----------CACCAGGTTGATTCTGCCTGACGTAG-ACGCTAGTCTCTGAGATTAAGCC
E. schubergi           ----------CACCAGGTTGATTCTGCCTGACGTAG-ACGCTAGTCTCTGAGATTAAGCC
N. corneum             ----------CACCAGGTTGATTCTGCCTGACGTAG-ATGCTAGTCTCTAAGATTAAGCC
E. bieneusi            ----------CACCAGGTTGATTCTGCCTGACGTAG-ATGCTAGTCTCTGAGATTAAGCC
A. penaei              ------------------------------------------------------------
G. atherinae           ----------CACCAGGTTGATTCTGCCTGACGTGG-ATGCTAGTCTCATAGGTTAAGCC
Ichthyosporidium sp.   ----------CACCAGGTTGATTCTGCCTGACGTGG-ATGCTAGTCTCTAAAGTTAAGCC
Vairimorpha sp.        ------------------------------------------------------------
A. michaelis           ---------------GGTTGATTCTGCCTGACGTAGAACGCTAGTCTCACAGATTCAGCC


                       61                                                       120
Thelohania sp.         -----------------------------------------------------------T
T. solenopsae          -----------------------------------------------------ATAACAT
N. bombycis            ATGCATGTTTATTGAATA----TAAAGA--------------------AAAGACGAACAG
N. trichoplusia        ATGCATGTTTATTGAATA----TAAAGA--------------------AAAGACGAACAG
V. necatrix            ATGCATGTTTTTGATA---------TGG--------------------AAAAATGGACTG
N. vespulae            ATGCATGTTTTTGACAT--------TTG--------------------AAAAATGGACTG
N. apis                ATGCATGTTTTTGACGTACTATGTACTG--------------------AAAGATGGACTG
E. hellem              ATGCATGTTTATGAAGCCTTTATGGGGG--------------------ATTGACGGACGG
S. intestinalis        ATGCATGTTGATGAA--CCTTGTGGGGG--------------------ATTGACGGACGG
E. cuniculi            ATGCATGCTTGTGAACTCTTTGTGGGGG--------------------ATTAGCGGACGG
Pleistophora sp.       ATGCATGTCTATGAAA-C------------------------------AAGGACGAACAG
E. schubergi           ATGCATGTCTATGGAA-C------------------------------AAGGACGAACAG
N. corneum             ATGCATGTTTCCGCAATC------------------------------AGGGACGAATAG
E. bieneusi            ATGCATGTCAGTGAAGCC-TTACGGTGG--------------------AACGGCGAACGG
A. penaei              ------------------------------------------------------------
G. atherinae           ATGCATGTGCAAGCGAAGCGTAAGTGGAGCGGCGCA---AGGCTCAGTAACGGGCGAGTA
Ichthyosporidium sp.   ATGGATGTCTAAGCAAAGCGTAAGTCGAGCGGCAC----AGGCTCAGTAACGGGCGAATA
Vairimorpha sp.        ------------------------------------------------------------
A. michaelis           ATGCAAGTAGTATGTATG-----------------------------TAATACACAATGG


                       121                                                      180
Thelohania sp.         AATCTACATAAATGGATAACCTTGTCA---AGATAAGGCTAATACAGTAAAGATGTTAGA
T. solenopsae          AATCTACATAAATGGATAACCTTGTCA---AGATAAGGCTAATACAGTAAAGATGTTAGA
N. bombycis            CTCAGTAACTCTTATTTGATTTGATGTA--TTAGGATTCTAACTATGTTAAATTATAG-G
N. trichoplusia        CTCAGTAACTCTTATTTGATTTGATGTA--TTAGGATTCTAACTATGTTAAATTATAG-G
V. necatrix            CTCAGTAATACTCACTTTATTTAATGTATTAAATTAGTATAACTGCGTTAAAGTGTAGCA
N. vespulae            CTCAGTAATACTCACTTTATTTTATGTA-CATTTGAAACTAACTACGTTAAAGTGTAG-A
N. apis                CTCAGTAATACTCACTTTATTTGATGTACCTTAT--ACATAACTACGTTAAAGTGTAGC-
E. hellem              CTCAGTGATAGTACGATGATTTGATTGGGAGCCTGGATGTAACTGTGGGAAACTGCAG-G
S. intestinalis        CTCAGTGATAGTACGATGATTTGGTTGGCGGGAGAGCTGTAACTGCGGGAAACTGCAG-G
E. cuniculi            CTCAGTGATAGCACGATGATTTGTTTGCGGGATGAGCAGTAGCTGCGGGAAACTGCAG-A
Pleistophora sp.       CTCAGTAAAACTGCGATGATTCAGTCTGTCTGTCAAGA-TAACCACGCGAAAGTGTGG-C
E. schubergi           CTCAGTAAAACTGCGATGATTCAGTCTGTCTGTCAAGA-TAACCACGCGAAAGTGTGG-C
N. corneum             CTCAGTAAAACTGCGATGATTTAGTCTGGCTGTGTAGA-TAACTACGTGAAAATGTAG-C
E. bieneusi            CTCAGTAATGTTGCGGTAATTTGGTCTCTGTGTGTAAACTAACCACGGTAACCTGTGG-C
A. penaei              ------------ACTTTTAACTAACCT-TTTGTACTAA-TAATTAAGGGAAACTGTAA-T
G. atherinae           TTTGATCTCCTAGAGTGGATATCCTCTGTAACCGGAGGGCA--AAACACAAGATGAGCGA
Ichthyosporidium sp.   TTTAATCTCCTCGAGTGGATATCCTCTGTAACCGGAGGGCA--AAACACAGGACGTGCAG
Vairimorpha sp.        ----------GGCGTACGGCTCAGT-----AGGACAGGGAAATCTAGCCACGAAGGAGGA
A. michaelis           CTCAGTATCG--AGTATAGCTTTGCTCTCCAAGATGTGATACTTTCAGGAAACAGAAA-A
                                                               *        *
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                        181                                                   240
Thelohania sp.          A-------------GCATGAAAGCGGAGCATCAATGTAGCGTTGGTTTCTGACCTATCAG
T. solenopsae           A-------------GCATGAAAGCGGAGCATCATTGTAGGATTGGTTTCTGACCTATCAG
N. bombycis             TAA---------------------------CAATAATACAATAAGAATAAGATCTATCAG
N. trichoplusia         TAA---------------------------CAATAATACAATAAGAATAAGATCTATCAG
V. necatrix             TAA---------------------------GACATATACAGTAAGAGTGAGACCTATCAG
N. vespulae             TAA---------------------------GATGTGTACAGTAAGAGTGAGACCTATCAG
N. apis                 TAA---------------------------CATATGTACAGTAAGAGTGAGACCTATCAG
E. hellem               TAAGTTCTGGGGGTGGTAGTTTGTAGCTACTGCGTACCGAGTAAGTTGTAGGCCTATCAG
S. intestinalis         TA-------GGGGGCTAGGAGTGTTTTTGACACGAGCCAAGTAAGTTGTAGGCCTATCAG
E. cuniculi             TA-------------GTGGTCTGCCCCTGTGGGTTGGCAAGTAAGTTGTGGGCCTATCAG
Pleistophora sp.        TAAG--------------------------AGGGGACAGAACAAGACGCAGGACTATCAG
E. schubergi            TAAG--------------------------AGGGGACAGAACAAGACGCAGGACTATCAG
N. corneum              TAAG--------------------------GGAAGGCAGAATAAGACGCAGGACTATCAG
E. bieneusi             TAAA--------------------------AGCGG--AGAATAAGGCGCAACCCTATCAG
A. penaei               TAAA--------------------------AATCATGAGGATGTGAGGTAGACCTATTAG
G. atherinae            TTGACGA---GGTCGTTCGTTTAACGAATAGTGTAGGAGAGTAAGAAGCCATCCCATCAG
Ichthyosporidium sp.    TTGTATAA-GGATTGTTCGTTTAAC-ATTAGTGGGGGAGAGTAAGACGCCAGTCCATCAG
Vairimorpha sp.         TAACCA------CGGTAAGCTGTGGCTAAAACGAGCGTGGGTGAGTTCTTGGCCTATCAG
A. michaelis            TAAAGCATCTATCTTCTAAAGTTTTTTAGAGGAGAGGAGAAGAAG-CACTCACCTATCAG
                                                                    *        * ** **

                        241                                                   300
Thelohania sp.          TTAGTATGTTTTGTAAGGGAGAACATAGACTATGACGGGTAACGGGGGATGCACGTCTGA
T. solenopsae           TTAGTATGTTTTGTAAGGGAGAACATAGACTATGACGGGTAACGGGGGATGCACGTCTGA
N. bombycis             TTAGTTGTTAAGGTAATGGCTTAACAAGACTATGACGGATAACGGTATTACTTTGTAATA
N. trichoplusia         TTAGTTGTTAAGGTAATGGCTTAACAAGACTATGACGGATAACGGTATTACTTTGTAATA
V. necatrix             CTAGTTGTTAAGGTAATGGCTTAACAAGGCAATGACGGGTAACGGTATTACTTTGTAATA
N. vespulae             CTAGTTGTTAAGGTAATGGCTTAACAAGGCAGTGACGGGTAACGGTATTACTTTGTAATA
N. apis                 CTAGTTGTTAAGGTAATGGCTTAACAAGGCAATAACGGGTAACGGTATTACTTTGTAATA
E. hellem               CTGGTAGTTAGGGTAATGGCCTAACTAGGCGGAGACGGGAGACGGGGGATCAGGGTTTGA
S. intestinalis         CTGGTAGTTAGGGTAATGGCCTAACTAGGCGGAGACGGGAGACGGGGGATCGGGGTTTGA
E. cuniculi             CTGGTAGTTAGGGTAATGGCCTAACTAGGCGCAGACGGGATACGGGGGATCAGGGTTTGG
Pleistophora sp.        TTAGTTGGTAGTGTAATGGACTACCAAGACGGTGACGGTTGACGGGGAATGAGGGTTCTA
E. schubergi            TTAGTTGGTAGTGTAATGGACTACCAAGACGGTGACGGTTGACGGGGAATGAGGGTTCTA
N. corneum              TTAGTTGGTAGTGTAATGGACTACCAAGACAGTGACGGTTGACGGGAAATTAGGGTTTTG
E. bieneusi             CTTGTTGGTAGTGTAAAGGACTACCAAGGCCATGACGGGTAACGGGAAATCAGGGTTTGA
A. penaei               CTAGTTGGTTGTGTAAAGGACTACCAAGGCTATAATGGGTAACGGAGATTTAGTGATCGA
G. atherinae            TTAGTAAGTAGGGTAAGGGCCTACTTAGACGAAGACGGGT-ACGGGGAATTATCGTTTGA
Ichthyosporidium sp.    TTAGTAAGTAGGGTAAGGGCCTACTTAGACGAATACGGAT-ACGGGGAATTATCGTTTGA
Vairimorpha sp.         CTAGTCGGTACGGTAAGGGCGTACCGAGGCAATAACGGGTAACGGGGAATCGGGGTTCGA
A. michaelis            TTAGTAGGTATGGTAAGGGCATACCTAGACGAAGACGGGT-ACGGGGAAGGCAACTTCGA
                         * **   *   **** **   *   ** *    * **   ****

                        301                                                   360
Thelohania sp.          TACCGGAGAGGAAGCCTT--AGAAACCGCTT--TCACGTC-CAAGGATGGCAGCAGGCGC
T. solenopsae           TACCGGAGAGGAAGCCTT--AGAAACCGCTT--TCACGTC-CAAGGATGGCAGCAGGCGC
N. bombycis             TTCCGGAGAAGGAGCCTG--AGAGATTGCT-TACTAAGTCATAAGGATTGCAGCAGGGGC
N. trichoplusia         TTCCGGAGAAGGAGCCTG--AGAGATTGC--TACTAAGTC-TAAGGATTGCAGCAGGGGC
V. necatrix             TTCCGGAGAAGGAGCCTG--AGAGACGGC--TACTAAGTC-TAAGGATTGCAGCAGGGGC
N. vespulae             TTCCGGAGAAGGAGCCTG--AGAGACGGC--TACTAAGTC-TAAGGATTGCAGCAGGGGC
N. apis                 TTCCGGAGAAGGAGCCTG--AGAGACGGC--TACTAAGTC-TAAGGATTGCAGCAGGGGC
E. hellem               TTCCGGAGAGGGAGCCTG--AGAGATGGCT--ACTACGTC-CAAGGATGGCAGCAGGCGC
S. intestinalis         TTCCGGAGAGGGAGCCTG--AGAGATGGCT--ACTACGTC-CAAGGATGGCAGCAGGCGC
E. cuniculi             TTCCGGAGAAGGAGCCTG--AGAGATGGCT--ACTACGTC-CAAGGACGGCAGCAGGCGC
Pleistophora sp.        TACCGGAGAGGGAGCCTG--AGAGATAGCT--CCCACGTC-CAAGGACGGCAGCAGGCGC
E. schubergi            TACCGGAGAGGGAGCCTG--AGAGATAGCT--CCCACGTC-CAAGGACGGCAGCAGGCGC
N. corneum              TACCGGAGAGGGAGCCTG--AGAGATTGCT--CCCACGTC-CAAGGACGGCAGCAGGCGC
E. bieneusi             TTCCGGAGAGGGAGCCTG--AGAGATGGCT--CCCACGTC-CAAGGACGGCAGCAGGCGC
A. penaei               AACCGGAGATGGAAGCTG--AGAAACGGTT--CCAATGTC-CAAGGATAGCAGCAGGCGC
G. atherinae            TTCCGGAGAGGGAGCCTG--AGAGACGGCT--ACCAGGTC-CAAGGACAGCAGCAGGCGC
Ichthyosporidium sp.    TTCCGGAGAGGGAGCCTG--AGAGACGGCT--ACCGGGTC-CAAGGACAACAGCAGGCGC
Vairimorpha sp.         TTCCGGAGAGGAAGCCTG--AGAAACGGCT--ACCACGTC-CAAGGAAGGCAGCAGGCGC
A. michaelis            TTCCGGAGAGGGCGCCTT-TAGAGATGGCG--ACCAGTTC-TAAGGAGTGCAGCAGGCTC
                          ******* *    **   *** *  *          **  *****   *******  *
                         361                                                      420
Thelohania sp.           GAAACTTAC--CCAATTATT-GTATTGATAGAGGTAGTTATGACGCATGTTAAGATTTTA
T. solenopsae            GAAACTTAC--CCAATTATT-GTATTGATAGAGGTAGTTATGACGCATGTTAAGATTTTA
N. bombycis              GAAACTTGA-CCTATG-ATA--TTAT-ATTGAGGCAGTTATGAGTAGTATTTTATAATTA
N. trichoplusia          GAAACTTGA-CCTATG-ATA--TTAT-ATTGAGGCAGTTATGAGTAGTATTTTATAATTA
V. necatrix              GAAACTTGA-CCTATGGAT--TTTA--TCTGAGGCAGTTATGGGAAGTAATATTCTATTG
N. vespulae              GAAACTTGA-CCTATGGAT--TTTA--TCTGAGGCAGTTATGGGAAGTAATATTATATTG
N. apis                  GAAACTTGA-CCTATGGAT--ATTA--TCTGAGGCAGTTATGGGAAGTAACAT--AGTTG
E. hellem                GAAACTTG--CCTAATCCT-TATT---GGGGAGGCGGTTATGAGAAGTAAGATGTT----
S. intestinalis          GAAACTTG--CCTAATCCT---TT---GGGGAGGCGGTTATGAGAAGTGAG-TTTT----
E. cuniculi              GAAACTTG--CCTAATCCT---TT---GGGGAGGCGGTTATGAGAAGTGATGGTGTGCGA
Pleistophora sp.         GAAAATTG--CCCACTGTT---T--G-GAGGAGGCAGTTATGAGACGTGAGAAAGAGTGC
E. schubergi             GAAAATTG--CCCACTGTT---T--G-GAGGAGGCAGTTATGAGACGTGAGAAAGAGTGC
N. corneum               GAAAATTG--CCCACTCTT---T--G-CAGGAGGCAGTTATGAGACGTGAAGATGAGTAT
E. bieneusi              GAAACTTG--TCCACTCCT---TACG-GGGGAGACAGTCATGAGACGTGAGTATAAGACC
A. penaei                GAAAATTG--CACACTCTT---TAAT-GGGGATGCAGTTATGAGGTATGACAGAAAGGGT
G. atherinae             GAAAATTACCGCAGCCTG----CATTCAGGGCGGTAGTAAGGAGACGTGAAAACAATGTG
Ichthyosporidium sp.     GAAAATTACCGCAGCCTG----CATTCAGGTCGGTAGTAAGGAGACGTGTAAACGATGTG
Vairimorpha sp.          GGAAATTAC--CCACTTG----GAGGACCAGAGGTAGTTATGGGCGTAAAGATGAGAAA
A. michaelis             GAAACTTA--CCGAATTATAGAATA-----GAGGTAGTGATGGAAACGTTTATATAGAAA
                         * ** **                             ** * *

                         421                                                      480
Thelohania sp.           AATTGAAACTTCATTAAAGATAGA--TAAGCGACTGGAGGGCAAG-TCTGGTGCCAGCAG
T. solenopsae            AATTGAAACTTCATTAAAGATGGT--TAAGCGACTGGAGGGCAAG-TCTGGTGCCAGCAG
N. bombycis              TTGTAGTATTGTAAGTACATATTACAAGATAAATCGGAGGGCAA-ATCGAGTGCCAGCAG
N. trichoplusia          TTGTAGTATTGTAAGTACATATTACAAGATAAATCGGAGGGCAA-ATCGAGTGCCAGCAG
V. necatrix              TT-TCATATTGTAAAAGTATATGAGGTGATTAATTGGAGGGCAA-ATCAAGTGCCAGCAG
N. vespulae              TT-TCATATTTTAAAAGTATATGAGGTGATTAATTGGAGGGCAA-ATCAAGTGCCAGCAG
N. apis                  TT-TCACATTTTAAACGTATGTGAGCAGATTAATTGGAGGGCAA-ATCGAGTGCCAGCAG
E. hellem                -TAGCA-----AGTATAAATTTGTTGTGATTTACTGGAGGGCAAG-TCGGGTGCCAGCAG
S. intestinalis          -TTTCG-----AGTGTAAAGGAGTCGAGATTGATTGGAGGGCAAG-TCGGGTGCCAGCAG
E. cuniculi              GTGCAA-----AGGGAATGGCTATTGTTGTATGTTGGAGGGCAAGCTCGGGTGCCAGCAG
Pleistophora sp.         TTGGTA-----AAGAGAAGCAGGAG------AATTGGAGGGCAAG-TTTGGTGCCAGCAG
E. schubergi             TT-GTA-----AAGAGAAGCAGGAG------AATTGGAGGGCAAG-TTTGGTGCCAGCAG
N. corneum               CTTGTA-----AAGAGGGATAGGAG------AATTGGAGGGCAAG-TTTGGTGCCAGCAG
E. bieneusi              TGAGTG-----TAAAGACCTTAGGGTGAAGCAATTGGAGGGCAAGCTTTGGTGCCAGCAG
A. penaei                TATCAA-----TAAATAAGATGACGTAAAGCTATTAGAGGGAAAG-TTTGGTGCCAGCAG
G. atherinae             CGGGCA--------AAAAACGCACTAGAT---ACAGGAGGACAAG-ACTGGTGCCAGCAC
Ichthyosporidium sp.     CAGGTA--------AAGAATGCACTGTAT---ACAGGAGGACAAG-ACTGGTGCCAGCAC
Vairimorpha sp.          AGTGTA--------AAAAGCTTTTTGAATGCGACTGGAGGGCAAG-TCTGGTGCCAGCAG
A. michaelis             TACTGGTAAAGCAAGTA-------------TTATTAACTGAGGAAAGCTGGTGCCAGCAG
                                                                *   *      *********

                         481                                                      540
Thelohania sp.           CCGCGGTAATTCCAGCTCCAGTAGTGCATAT-----ACATGCTGTAGTTAGAAAGTTTGT
T. solenopsae            CCGCGGTAATTCCAGCTCCAGTAGTGCATAT-----ACATGCTGTAGTTAGAAAGTTTGT
N. bombycis              CCGCGGTAATACTTGTTCCGATAGTGTGTATG--ATGATTGATGCAGTTAAAAAGTCTGT
N. trichoplusia          CCGCGGTAATACTTGTTCCGATAGTGTGTATG--ATGATTGATGCAGTTAAAAAGTCTGT
V. necatrix              CCGCGGTAATACTTGTTCCAAGAGTGTGTATG--ATGATTGATGCAGTTAAAAAGTCCGT
N. vespulae              CCGCGGTAATACTTGTTCCAAGAGTGTGTATG--ATGATTGATGCAGTTAAAAAGTCCGT
N. apis                  CCGCGGTAATACTTGTTCCAAGAGTGTGTATG--ATGATTGATGCAGTTAAAAAGTCCGT
E. hellem                CCGCGGTAATACCTGCTCCAGTAGTGTCTATG--GTGAATGCTGCAGTTAAAATGTCCGT
S. intestinalis          CCGCGGTAATACCTGCTCCAATAGTGTCTATG--GTGAATGCTGCAGTTAAAAAGTCCGT
E. cuniculi              CCGCGGTTAATTGAATCCTGCCAATTGGGTTG--ATGGATGCTGCCGTTAAAATGTCCGT
Pleistophora sp.         CCGCGGTAATACCGACTCCAAGAGTGTGTATG--AGAGATGCTGCAGTTAAAAAGTCCGT
E. schubergi             CCGCGGTAATACCGACTCCAAGAGTGTGTATG--AGAGATGCTGCAGTTAAAAAGTCCGT
N. corneum               CCGCGGTAATACCGACTCCAAGAGTGTGTATG--AGAGATGCTGCAGTTAAAAAGTCCGT
E. bieneusi              CCGCGGTAACTCCAACTCCAAGAGTGTCTATG--GTGGATGCTGCAGTTAAAGGGTCCGT
A. penaei                CCGCGGTAATACCAACTCTAAGAGTCTCTATG--CGAGTTGCTGCAGTTAAAAAGTCCGT
G. atherinae             CCGCGGTAATACCAGCTCCTGGAGTGTCTATGATATGATTGCTGCAGTTAAAGAGTTCGT
Ichthyosporidium sp.     CCGCTGTAATACCAGCTCCTGGAGTGTCTATG--ATGATTGCTGCAGTTAAAGCGTTCGT
Vairimorpha sp.          CCGCGGTAATTCCAGCTCCAGGAGCTTCTGTGTGA--GTTGCTGCGGTTAAAAAGTGCGT
A. michaelis             CCGCGGTAATACTTGCTCCAGGAGCTTATTCG----ATATGTTGCGGTTAAAACGTCCGT
                         **** ** *        *    *                ** **  **** *  **  **
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                        541                                                    600
Thelohania sp.          AGCCAGTTTATGGATT-GTTTTTGATAATAGTTATTCTCCAAAAGAGCTAATTTTAACTA
T. solenopsae           AGCCAGTTTATGGATT-GTTTTTGATAATAGTTATTCTCCAAAAGAGCTAATTTTAACTA
N. bombycis             AGTTT-A-T---------------------------------------------------
N. trichoplusia         AGTTT-ATT---------------------------------------------------
V. necatrix             AGTTT-ATA---------------------------------------------------
N. vespulae             AGTTT-ATA---------------------------------------------------
N. apis                 AGTTT-ATT---------------------------------------------------
E. hellem               AGTTG-TTTGTATGT-----CTTTTGAGTGATGTTTATGGTTTTTAGTGGATGTAG-TTT
S. intestinalis         AGTCT-TTTGTATGT-----CTTTGTTTGGGGGATTATGTCCTGATGTGGATGTAA-GAG
E. cuniculi             AGTCT-GTTGTGTATG-TC-TTTGTGTGTGATGTTTGTGGTTGTGTGTGGATGTAGTGAT
Pleistophora sp.        AGTCG-TGGAGACG----------GAAAGAGAG--GCGGAGCCTCTTTGAGAT-------
E. schubergi            AGTCG-TGGAGACG----------GAAAGAGAG--GCGGAGCCTCTTTGAGAT-------
N. corneum              AGTCA-TAGAAGGG----------CAAAGAGAGATGCGAGGTCTCACAGTGCG-------
E. bieneusi             AGTCG-TGAATGCA--------ATTAAATGTCGTTGTTCAATAGCGATGAGTTTGCTA--
A. penaei               AGTC--TTTACGTA--------ATTAAAAATGAATGATCAAGTTTCATATTTTTACGTTT
G. atherinae            AGTCGAAGTGGTTATA-ACGGTGTAACAGGCCTTCTCTCAAGGAGGGTTATGCGCCGTGA
Ichthyosporidium sp.    AGTCGAACCGGGTTGA-ATTGCGTGACAGTCAGACTCTCAAGGTGTGATGAGCGCTGTGA
Vairimorpha sp.         AGTCG--GGGAGCAGG-CCAGCAGAAAAGGTGGGGAATCACGCCTAGCATAGTGCAGGAA
A. michaelis            AGTCGCGGCTGGGA---CTGACCTGTAATCTATTTGGTCAACAGATAGATAGGGGCAGTA
                        **

                        601                                                    660
Thelohania sp.          ATTCATAA---AAATAGAAGCGGATGAAGGTAATTGTATTCACCAGCAAGAGGTAAAATT
T. solenopsae           ATTCATAA---AAATAGAAGCGGATGAAGGTAATTGTATTCACCAGCAAGAGGTAAAATT
N. bombycis             TTAT---------AATAAGGATTGTAAGGTATACTGTATGGTTAGGAGAGAGATGAAATG
N. trichoplusia         TTAT---------AATAAGCATTGTAAGGTATACTGTATGGTTAGGAGAGAGATGAAATG
V. necatrix             TT-T---------AAGAAGCAATATGAGGTGTACTGTATAGTTGGGAGAGAGATGAAATG
N. vespulae             TT-T---------AAGAAGCAATATGAGGTGTACTGTATAGTTGGGAGAAAGATGAAATG
N. apis                 AT-T---------AAGAAGCAATATGAGGTGTACTGTATAGTTGGGAGAGAGATGAAATG
E. hellem               TATT---------GTAGCAGAGGACGAGGGGCACTGGATAGTTGGGCGAGGGGTGAAATA
S. intestinalis         GTTT---------G--GCAGAGGACGAGGGGCACCGGATAGTTGGGCGAGGGGTGAAATA
E. cuniculi             GTGT---------GTGGCAGAGGACGAGGGGCACTGGATAGTTGGGCGAGAGGTGAAATG
Pleistophora sp.        GCTCT--------GGAGAAGCCAACAGGGGGCACAGTATACCAGGGCGAGAGATGAAATG
E. schubergi            GCTCT--------GGAGAAGCCAACAGGGGGCACAGTATACCAGGGCGAGAGATGAAATG
N. corneum              ATGAT--------GGAGGAGCCGATGGGGAACATAGTATACCAGGGCGAGAGATGAAATG
E. bieneusi             ATGTT--------TGCGGAACGGATAGGGAGTGTAGTATAGACTGGCGAAGAATGAAATC
A. penaei               ATA----------TATGAGACGGATTGGGAGCATAGTATAACTGGGTTAAGAATGAAATC
G. atherinae            TTCCATGG---AATAAGGAGCGTTTAGGGGCCAGGTTATTAAGCGACGAGGGGTGAAATC
Ichthyosporidium sp.    TTCTGGGG---AATAAGGAGTGTTTAGGGGCCAGGGTATTAAACGGCAAGCGGTGAAATG
Vairimorpha sp.         --CTGGGA---CCTAGGGACCGGAGAGGGGCAACCTAATTCTTGGGCGAGGGGTGAAAAC
A. michaelis            GCAAGTTG----GAAAAGAGCAATTTGGTGTCAGCTAATGGTATGGGGAGGGGTAAAGTC
                                                             **         *    * **

                        661                                                    720
Thelohania sp.          TGATGAC-CTGGTGAGGACATTCAGAGGCGAAAGCGATTGCCTAGTACATTTTTGAT---
T. solenopsae           TGATGAC-CTGGTGAGGACATTCCGAGGCGAAAGCGATTGCCTAGTACGTTTTTGAT---
N. bombycis             TGATAACCCTAAC-TGGATGAACAGAAGCGAAAGCTGTATACTTAAATGTATTATTA---
N. trichoplusia         TGATAACCCTAAC-TGGATGAACAGAAGCGAAAGCTGTATACTTAAATGTATTATTA---
V. necatrix             TGACGACCCTGAC-TGGACGAACAGAAGCGAAAGCTGTACACTTGTATGTATTTTTT---
N. vespulae             TAACGACCCTGAC-TGGACGAACAGAAGCGAAAGCTGTACACTTGTATGTATTTTTT---
N. apis                 TGACGACCCTGAC-TGGACGAACTGAAGCGAAAGCTGTACACTTGTATGTATTTTTT---
E. hellem               CGAAGACCCTGAC-TGGACGAAGAGAAGCGAAGGCTGTGTTCTTGGACTTTTGTGGT---
S. intestinalis         CGAAGACCCTGAC-TGGACGGACAGAAGCGAAGGCTGTGCTCTTGGACTTATGTGAC---
E. cuniculi             CGAAGACCCTGAC-TGGACGAGCGGAAG---AGGCTGTGCTCTTGGACTAATGTTGTTGC
Pleistophora sp.        CCAAGACCCCTGG-TGGACTGAGCGAGGCGAAAGCGGTGCTCTTGTGGGTGTTCGGT---
E. schubergi            CCAAGACCCCTGG-TGGACTGAGCGAGGCGAAAGCGGTGCTCTTGTGGGTGTTCGGT---
N. corneum              CCAAGACCCCTGG-TGGACTGAGCGAGGCGAAGGCGATGTTCTTGTAGGCATTCGGT---
E. bieneusi             TCAAGACCCAGTT-TGGACTAACGGAGGCGAAGGCGACACTCTTAGACGTATCTTAG---
A. penaei               TCACTACCCTAGT-TGGACTATCAGAAGCGAAAGCGATGCTCTAATACGTACTTTTA---
G. atherinae            TGGTGACTCGCTTA-GGAGCAACAGAGGCGAAAGCGCTGGCCAGGAGCGAATCCGAT---
Ichthyosporidium sp.    TGTTGACCCGTTTATGGAGCGACAGAGGCGAAAG-GCTGGCCAGGGGCAAATCCGAT---
Vairimorpha sp.         TGCTGACCCTGAGA-GGAGGAACAGAGGCGAAGGCGGTTGTCCGGGACGGGTCTGAC---
A. michaelis            TGAGGATCCTG--CAGGAGGAGCAAAGGCGTAAGCACTGACAAAGATTGATTCTGTT---
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                       721                                                       780
Thelohania sp.         GGTAAAGAACGTAAGCCGGAGGATCAAAGATGATTAGATACCGTTGTAGTTCCGGCCGTA
T. solenopsae          GGTAAAGAACGTAAGCCGGAGGATCAAAGATGATTAGATACCGTTGTAGTTCCGGCCGTA
N. bombycis            GAACAAGGACGTAAGCTAGAGGATCGAAGATGATTAGATACCATTGTAGTTCTAGCAGTA
N. trichoplusia        GAACAAGGACGTAAGCTAGAGGATCGAAGATGATTAGATACCATTGTAGTTCTAGCAGTA
V. necatrix            GAACAAGGACGTAAGCTGGAGGAGCGAAGATGATTAGATACCATTGTAGTTCCAGCAGTA
N. vespulae            GAACAAGGACGTAAGCTGGAGGAGCGAAGATGATTAGATACCATTGTAGTTCCAGCAGTA
N. apis                GAACAAGGACGTAAGCTGGAGGATCGAAGATGATTAGATACCATTGTAGTTCCAGCAGTA
E. hellem              GATGAAGGACGAAGGCTAGAGGATCGAAATCGATTAGATACCGTTTTAGTTCTAGCAGTA
S. intestinalis        GATGAAGGACGAAGGCTAGAGGATCGAAATCGATTAGATACCGTTTTAGTTCTAGCAGTA
E. cuniculi            GATGAAGGACGAAGGCTAGAGGATCGAAAACGATTAGATACCGTTTTAGTTCTAGCAGTA
Pleistophora sp.       GATCAAGGACGAAGGCTGGAGGATCGAAAGTGATTAGATACCGCTGTAGTTCCAGCAGTA
E. schubergi           GATCAAGGACGAAGGCTGGAGGATCGAAAGTGATTAGATACCGCTGTAGTTCCAGCAGTA
N. corneum             GATCAAGGACGAAGGCTGGAGTATCGAAAGTGATTAGATACCGCAGTAGTTCCAGCAGTA
E. bieneusi            GATCAAGGACGAAGGCAGGAGTATCGAAAGTGATTAGACACCGCTGTAGTTCCTGCAGTA
A. penaei              GATAAAGGACGAAGGCTAGAGTAGCGAAAGGGATTAGATACCCCTGTAGTTCTAGCAGTA
G. atherinae           GATAAAGGACGTAGGCTAGAGGATCGAAGACGATTAGAGACCGTTGTAGTTCTAGCAGTA
Ichthyosporidium sp.   GATAAAGGACGTAGGCTAGAGGATCGAAGACGATTAGAGACCGTTGTAGTTCTAGCAGTA
Vairimorpha sp.        GATCAAGTACGTGAGCAGGAGGATCAAAGACGATTAGACACCGTCGTAGTTCCTGCAGTA
A. michaelis           GATCAAGGACAGAGGCTAGAGGATCGAATACGATTAGATACCGTAGTAGTTCTAGCAGTG
                       *   *** **    **  *** * * **   ******* ***    ******  ** **

                       781                                                       840
Thelohania sp.         AATTATGCCAACTTGCA-TTTTGTTATT---TATACAAGGAGCATAGAGAAATTAAGAGT
T. solenopsae          AATTATGCCAACTTGCA-TCTTGTTATT---TATACAAGGAGCATAGAGAAATTAAGAGT
N. bombycis            AACTATGTTGAACCATAGATATATTTTG---ATATATATTTATGTAGAGAAATTAAGATT
N. trichoplusia        AACTATGTTGAATCATAGATATATTTTG---ATATATATTTATGTAGAGAAATTAAGATT
V. necatrix            AACTATGCCGACGATGTGATATGATATT---AATTGTATTAGATGATAGAAATTT-GAGT
N. vespulae            AACTATGCCGACGATGTGATATGATATA---TTTTGTATTACATAATAGAAATTA-GAGT
N. apis                AACTATGCCGACGATGTGATATGAGAT-----GTTGTATTACATTATAGAAATTA-GAGT
E. hellem              AACGATGCCGACTGGACG-GGACTGTT-----TTAGTGTTGTCCGAGAGAAATCTTAAGT
S. intestinalis        AACGATGCCGACTGGACG-GGACT-AT-----ATAGTGTTGTGCATGAGAAATCTTGAGT
E. cuniculi            AACGATGCCGACTGGACG-GGTCAGTG-----TGTG---TTGCCATGAGAAATCTTGAGT
Pleistophora sp.       AAAGATGCCGACATGC--TCGG---TG-----GCAACACGGGGCGGGGAGAAATCTTAGA
E. schubergi           AAAGATGCCGACATGC--TCGG---TG-----GCAACACGGGGCAGGGAGAAATCTTAGA
N. corneum             AAAGATGCCGACATGC--TCAT---TG-----GACACAGTGGGCAGGGAGAAATCTTAGA
E. bieneusi            AACTATGCCGACAGCCTGTGTG---TG-----AGAATACGTGGGCGGGAGAAATCTTAGT
A. penaei              AACTATGCCGACAGAATGTTAGATATA-----TTTCTAGTGTTCAAGGGAAACCTTAAGT
G. atherinae           AACGATGCCGATACCGTGGTGCG---------GATACGCGACGCGGAAGAGAAATCGAGT
Ichthyosporidium sp.   AACAATGCCGATGTTGTGGTGCC---------GTAACG-GACGCAAAAGAGAAATCTAGT
Vairimorpha sp.        AACGATGCCGACGGGGCAGCAGG--------GGAACTTGTTGCCTGAGGGAAACCA-AGT
A. michaelis           ACCGATGATGATTTTGCCTTATGCAAT--------------------AGAGAAATCAAAT
                       *   ***   *                                        *     *

                       841                                                       900
Thelohania sp.         TTTTGGGCTCTAGGGATAGTAATCCGGCAACGGACAAACTTAAA--GAAATTGGCGGAAG
T. solenopsae          TTTTGGGCTCTAGGGATAGTAATCCGGCAACGGACAAACTTAAA--GAAATTGGCGGAAG
N. bombycis            ATATTGACTCTGGGGATAGTATGATCGCAAGATTGAAAATTAAA--GAAAGTGACGGAAG
N. trichoplusia        ATATTGACTCTGGGGATAGTATGATCGCAAGATTGAAAATTAAA--GAAATTGACGGAAG
V. necatrix            TTTTTGGCTCTGGGGATAGTATGATCGCAAGATTGAAAATTAAA--GAAATTGACGGAAG
N. vespulae            TTTTTGGCTCTGGGGATAGTATGATCGCAAGATTGAAAATTAAA--GAAATTGACGGAAG
N. apis                TTTTTGGCTCTGGGGATAGTATGATCGCAAGATTGAAAATTAAA--GAAATTGACGGAAG
E. hellem              ATGTGGGTTCTGGGGATAGTATGCTCGCAAGAGTGAAACTTGAA--GAGATTGACGGAAG
S. intestinalis        ATGTGGGTTCTGGGGATAGTATGCTCGCAAGAGTGAAACTTGAA--GAGATTGACGGAAG
E. cuniculi            ATGCGGGTTCTGGGGATAGTATGCTCGCAAGAGTGAAACTTGAA--GAGATTGACGGAAG
Pleistophora sp.       GTTCGGGCTCTGGGGATAGTATGCTCGCAAGGGTGAAAATTAAA--GAAATTGACGGAGC
E. schubergi           GTTCGGGCTCTGGGGATAGTATGCTCGCAAGGGTGAAAATTAAA--GAAATTGACGGAGC
N. corneum             GTTCGGGCTCTGGGGATAGTATGCTCGCAAGGGTGAAAATTAAA--GAAATTGACGGAGC
E. bieneusi            GTTCGGGCTCTGGGGATAGTACGCTCGCAAGGGTGAAACTTAAAGCGAAATTGACGGAAG
A. penaei              GATCGGGCTCTGGGGAGAGTATGCTCGCAAGTGTGAAAATTAAA-CGAAATTGACGGAGT
G. atherinae           ---AGGGCCCTGGGGAGAGTACACGCGCAAGCGAGAAATTTAAAG-GAAATTGACGGAAG
Ichthyosporidium sp.   ---AGGGCCCTGGGGAGAGTACACGCGCAA-CAGGAAATTTAAAG-GAAATTGACGGAAG
Vairimorpha sp.        GTACGGGCTCCGGGGATAGTACGGGCGCAAGCTTGAAACTTAAA--GAAATTGACGGAAG
A. michaelis           A---GATCTCCGGGGAGTACATGCGCACAGGAACTTAA---------GAATTGACGGAAG
                                *  ****    *      **       **           * ** ****
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                        901                                                   960
Thelohania sp.          GACACCACAAGGAGTGGATTATACGGCTTAATTTGACTCAACGCGGGAAAACTTACCAGG
T. solenopsae           GACACCACAAGGAGTGGATTATACGGCTTAATTTGACTCAACGCGGGAAAACTTACCAGG
N. bombycis             AATACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACCCGGGGTAATTTACCAGG
N. trichoplusia         AATACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGGGGTAATTTACCAGG
V. necatrix             AATACCAGAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGTAACTTACCAAT
N. vespulae             AATACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGTAACTTACCAAT
N. apis                 AATACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGTAACTTACCAAT
E. hellem               GACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAACGCGGGGCAACTTACCGGT
S. intestinalis         GACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAACGCGGGGCAACTTACCGGT
E. cuniculi             GACACCACAAGGCGTGGAGTGTGCGGCTTAATTTGACTCAACGCGGGGCAACTTACCGGC
Pleistophora sp.        TACACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGAAGCTTACCAGG
E. schubergi            TACACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGAAGCTTACCAGG
N. corneum              TACACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGAAACTTACCAGG
E. bieneusi             GACACTACCAGGAGTGGATTGTGCTGCTTAATTTAACTCAACGCGGGAAAACTTACCAGG
A. penaei               TACACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGAATTTTACCAGG
G. atherinae            AACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAACGCGGGACAGCTTACCAGG
Ichthyosporidium sp.    AACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAACGCGGGACACCTTACCGGG
Vairimorpha sp.         GACACCACAAGGAGTGGAGTGTGCGGGTTAATTTGACTCAACGCGGGACAACTTACCGGG
A. michaelis            CTAGCCACAAGGGTTGGATTGTGCGGCTTAATTTGACTCAAAGCGGAAAAGCTTACCAAG
                            * *  ***  **** * * * * ******* ******  **    *  *****

                        961                                                  1020
Thelohania sp.          GCCTATGTATAAGAGAAAGTTAACATTGTATG-------TATACTTGATTGTACTTTGAG
T. solenopsae           GCCTATGTATAAGAGAAAGTTAACATTGTATG-------TATACTTGATTGTACTTTGAG
N. bombycis             TATAA---------------CATGGTATAATATTTT--------------ATCATGATAG
N. trichoplusia         TATAA---------------CATGATATAATATTTT--------------ATCATGATAG
V. necatrix             ATT----------------TTATTCAGAGAAGATTTTC--GATC-TGAGAATGATAATAG
N. vespulae             ATTTT-------------ATTATTTTGAGACGATTTTT--AATC-AGAGAATGATAATAG
N. apis                 ATTTT-------------ATTGTTCTGCGAGGATAT----GATC-TGAGGATGATAATAG
E. hellem               TCTGA--------------AGTGAGTGTGAGAGTGTTTTTACAT-GAT-GCTTACGGCGG
S. intestinalis         TCTGA--------------AGCGGGCAGGAGAACG--AGGACGG-GAT-GCGCGCGGCGG
E. cuniculi             TCTGA---------------AGGAATGCCTGTGAGGCATGGCAT-TG--GCATGCGGCGG
Pleistophora sp.        GCCAA----------------GTGCTGTGGAGAAAG----------GAGCAGGACAGAAG
E. schubergi            GCCAA----------------GTGCTGTGGAGAAAG----------GAGCAGTACAGAAG
N. corneum              GCCAA----------------GTATTGTGTAGAAAC----------GAGCAATACAGGAG
E. bieneusi             GTCAA----------------GTCATTCGTTGATCG---AATACGTGAGAATGGCAGGAG
A. penaei               GCTGA----------------ATATATTTGAGATTG---AATACATGAAATATATTTGAG
G. atherinae            CCCGACGGCCGGACGAGTGTTGTACACGATAGGTCGA-------------------AGAG
Ichthyosporidium sp.    CCC-ACGGCCACACGAGTGTGACACACGATA-GCCGA-------------------GGAG
Vairimorpha sp.         GCAGGCGACGAGAAGCGAAGGATGATGAAGAGATTC---ACAGACTGATTGCGTCGCGTG
A. michaelis            CTTATTTATTCAACGA--GTATTTATCCGAGAGTA--------------------AAATG
                                                                                   *

                        1021                                                 1080
Thelohania sp.          TGGTGCATGG-CCGTTTTCAACACGTGGGGTGACTTGTCAGGTTTATTCCGGTAACGTGT
T. solenopsae           TGGTGCATGG-CCGTTTTCAACACGTGGGGTGACTTGTCAGGTTTATTCCGGTAACGTGT
N. bombycis             TGGTGCATGG-CCGTTTCCAATGGATGCTGTGAAGT-AATGATTAATTTCAACAAGATGT
N. trichoplusia         TGGTGCATGG-CCGTTTCCAATGGATGCTGTGAAGT-AATGATTAATTTCAACAAGATGT
V. necatrix             TGGTGCATGG-CCGTTTTCAATGGATGCTGTGAAGT-TTTGATTAATTTCACCAAGACGT
N. vespulae             TGGTGCATGG-CCGTTTTCAATGGATGCTGTGAAGT-TTTGATTAATTTCAACAAGACGT
N. apis                 TGGTGCATGG-CCGTTTTCAATGGATGCTGTGAAGT-TTTGATTAATTTCAACAAGACGT
E. hellem               TGGTGCATGG-CCGTTTTAAATGGATGGCGTGAGCT-TTGGATTAAGTTACGTAAGATGT
S. intestinalis         TGGTGCATGG-CCGTTTGAAATGGATGGCGTGAGCT-TTGGATTAAGTTGCGTAAGATGT
E. cuniculi             TGGTGCATGG-GCCTTTTAAATGGATGGCGTGA-CT-TTGTCTTAAGTTGCGTAAGATGT
Pleistophora sp.        TGGTGCATGG-TCGTTGGAAATTGATGGGATGACTT-TGGCCTTAAATGGCTGAATGAGT
E. schubergi            TGGTGCATGG-TCGTTGGAAATTGATGGGATGACTT-TGGCCTTAAATGGCTGAATGAGT
N. corneum              TGGTGCATGG-TCGTTGGAAATTGATGGGATGACTT-TGACCTTAAATGGTTGAATGAGT
E. bieneusi             TGGTGCATGG-CCGTTGGAAATTGATGGGGCGACCT-TTAGCTTAAATGCTTAAACCAGT
A. penaei               TGGTGCATGG-TCGTTGTAAACTCATGGATTGATCT-TAAGTTCAACTGCTAAAATGGGT
G. atherinae            TGGTGCATGG-CCGTTAACGACGAGTGAGGTGACTT-TTGGGTTAAATCCGGGAAGTAGT
Ichthyosporidium sp.    TGGTGCATGGCCCGTTAACGACAAGTGA-GTGATCT-TTGGGTTAAGTCCGTAAATTAGT
Vairimorpha sp.         TGGTGCATGG-CCGTTTTTAACACGTGGGGTGACTTGTCAGGTTAAATCCGATAACGCGT
A. michaelis            GTGTGCATGG-CCGTTCCTAACACATGGAGTGATTTTGTGATTAACCTTCCGTAATCTGT
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                         1081                                                      1140
Thelohania sp.           GATGTGCAGTATGC----AACTAATGTTGTGAGACTTCTTGCGGTAAGC---TTGATGAA
T. solenopsae            GATGTGCAGTATGCAACTAACTAATGTTGTGAGACTTCTTGCGGTAAGC---TTGATGAA
N. bombycis              GAGACCCTCATTTAGACAGATGTAGTG----------ATACA----------TATGAAGG
N. trichoplusia          GAGACCCTCATTTAGACAGATGTAGTG----------ATACA----------TATGAAGG
V. necatrix              GAGACCCTTTTATTAATAGACAGACAC----------AATCAGTG-------TAGGAAGG
N. vespulae              GAGACCCTTTTATT-ATAGACAGACAC----------AATCAGTG-------TAGGAAGG
N. apis                  GAGACCCT----TTATTAGACTGACAC----------TATTAGTG-------TAGGAAGG
E. hellem                GAGACCCT--TTTTGACTGTGCTCTA------------TGGGCA---------AGGGAGG
S. intestinalis          GAGACCC----TTTGACAGTGCTCTT------------TGGGCA---------AGGGAGG
E. cuniculi              GAGACCC----TTTGACGGTGTTCTA------------CGAAGCA--------A-GGAGG
Pleistophora sp.         GAGATC---TTTGGACATG--TTCCC--------------ACAGGAA------CAGGAAGG
E. schubergi             GAGATC---TTTGGACATG--TTCCC--------------AC-GGAA------CAGGAAGG
N. corneum               GAGATCT--TTTGGACATG--TTCCG-------------CAC-GGAA------CAGGAAGG
E. bieneusi              GAGACCT--CCTTGACAGG--TGTTC-------------TGTAACA-------CAGGAGGG
A. penaei                GAGACTT--TCATAAACAGCTATCTA--------------ACAGGTA------GAGGAAGG
G. atherinae             GAGACCCCTACCGAAAGGACAGGTGC-----------CGAAAGCA-------CAGGAAGG
Ichthyosporidium sp.     GAGACCC---CAGCAAAGGACAGGTGC-----------GCAAAGCA-------CAGGAAGG
Vairimorpha sp.          GAGACCCTGTGTAGATGGAAATA-CGACGGGACATGGCAAGTGT--------CAGGAAGA
A. michaelis             GTAAATC--CTCATAATAGCTTGTTTGA-----------------------AAAGAACAA
                         *

                         1141                                                      1200
Thelohania sp.           GAGGCGCTATAACAGGTCAGTGATGCCCTTAGATGTTCTGGGCTGCACGTGTAATACAGT
T. solenopsae            GAGGCGCTATAACAGGTCAGTGATGCCCTTAGATGTTCTGGGCTGCACGTGTAATACAGT
N. bombycis              AGAGGATTAAAACAGGTCCGTTATGCCCTAAGATAATCTGGGTTGCACGCGCAATACAAT
N. trichoplusia          AGAGGATTAAAACAGGTCCGTTATGCCCTAAGATAATCTGGGTTGCACGCGCAATACAAT
V. necatrix              AAAGGATTAAAACAGGTCCGTTATGCCCTCAGACATTTTGGGCTGCACGCGCAATACAAT
N. vespulae              AAAGGATTAAAACAGGTCCGTTATGCCCTCAGACATTTTGGGCTGCACGCGCAATACAAT
N. apis                  AAAGGACTAAAACAGGTCAGTTATGCCCTCTGACATTTTGGGCAGCACGCGCAATACAAT
E. hellem                AATGGAACAGAACAGGTCCGTTATGCCCTGAGATGAAGCGGGCGGCACGCGCACTACGAT
S. intestinalis          AATGGAACAGAACAGGTCCGTTATGCCCTGAGATGAAGCGGGCGGCACGCGCACTACGAT
E. cuniculi              GATGGAAGAGAACAGGTCCGTTATGCCCTGAGATGAGGCGGGCTGCACGCGCAACTAGAT
Pleistophora sp.         -GGAGGCTATAACAGATCAGAGATGCCCTTAGATGCCCTGGGCTGCACGCGCAATACAAT
E. schubergi             -GGAGGCTATAACAGATCAGAGATGCCCTTAGATGCCCTGGGCTGCACGCGCAATACAAT
N. corneum               AAAAGGCTATAACAGATCCGAGATGCCCTCAGATGCCCTGGGCTGCACGCGCAATACAAT
E. bieneusi              TGGAGGCTATAACAGGTCCGTGATGCCCTTAGATATCCTGGGCAGCAAGCGCAATACAAT
A. penaei                GGAAGGCGATAACAGATCCGTGATGCCCTCAGATGTCCTGGGCTGCACGCGCAATACATT
G. atherinae             AAGGGTCAAGAACAGGTCAGTGATGCCCTCAGATGGTCTGGGCTGCACGCGCACTACAGT
Ichthyosporidium sp.     ATGGGTCAAGGACAGGTCAGTGATGCCCTTAGATGGTCCGGGCTGCACGCGCACTACAGT
Vairimorpha sp.          GCGGGTCGATAACAGGTCTGTGATGCCCGCAGATGTTCCGGGCGCCACGCGCACTACATT
A. michaelis             TTCGAGCAAGAACAGGTCAGTGATGTCCTTTGATAGCTTGGGCTGCACGCGCAATACAAT
                                 *  **** ** *  *** **   **      ***   ** * * *      *

                         1201                                                      1260
Thelohania sp.           GGGTATTTCAATATTTAATAGGA-GTAAATTTACCCGAGACAGGGATCATGCTTTGTAAG
T. solenopsae            GGGTATTTCAATATTTAATAGAA-GTAAATTTACCCGAGACAGGGATCATGCTTTGTAAG
N. bombycis              AAT-ATTTG-ATAT-----TATA------------------AGGGATAATATAATGTAAG
N. trichoplusia          AAT-ATTTG-ATAT-----TATA------------------AGGGATAATATAATGTAAG
V. necatrix              AGATATAT-AATC------TTTA------------------TGGGATAATATTTTGTAAG
N. vespulae              AGATATAT-AATC------TTTA------------------TGGGATAATATTTTGTAAG
N. apis                  AGA-CTTT-AATC------TTTA------------------TGGGATAATATTTTGTAAG
E. hellem                AGATGCCT-----------------ATGTGGGCTACTGTGA-GGGATGAAGCTGTGTAAT
S. intestinalis          AGATGGCG-----------------AGGGAGCCTGCTGTGA-GGGATGAAGCTGTGTAAT
E. cuniculi              AGATGGCG-----------------CTTCTGCCTGCTGTGAGGGGATGAAGCTGTGTAAG
Pleistophora sp.         AGCACGTA-GACG------TACAGAACAACACGTGCT-GAGGTGGACTGTGCTCTGCAAG
E. schubergi             AGCACGTA-GACG------TAGAGAACAACACGTGCT-GAGGTGGACTGTGCTCTGCAAG
N. corneum               AGCAGGTA-GAGA------GAGAGACAGGAAGGTGCT-CAGATGGACTATGTGCTGTAAG
E. bieneusi              ATCTCTTC-AGTA------GACAAAGTGATTTGAGAT-GAGTAGGATCTACGTTTGTAAA
A. penaei                ATGTATAT-TTCT------TATAAATAGATACTACATATTGGGGAATTGACTTTTGTAAA
G. atherinae             GGTCATAG--AAATGAAACGATAGAATTAAAGATGATCGAGAGGGAATGAGCTTTGTAAG
Ichthyosporidium sp.     GGTCGCCG--AAATTTAGATATAGAGCTAAAGGCGATCGAGAGGGAATGAGCTTTGGAAG
Vairimorpha sp.          GGACGGCG--ATATATGAAAAT--GAGGAGCCGTCCGTGGTTGGGATTGACGCTTGTAAT
A. michaelis             TTTTATGT-------------------AGTAAGATATAGATAGGGATTGAGGGCTGAAAG
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                          1261                                                       1320
Thelohania sp.            AAG---------------------------------------------------------
T. solenopsae             AAG---------------------------------------------------------
N. bombycis               ATATATTTGAACATGGAATTGCTAGTAAATTTT-ATTTAATAAGTAGAATTGAATGAGTC
N. trichoplusia           ATATATTTGAACATGGAATTGCTAGTAAATTTT-ATTTAATAAGTAGAATTGAATGAGTC
V. necatrix               AGATATTTGAACTTGGAATTGCTAGTAAATTTT-ATTAAATAAGTAGAATTGAATGTGTC
N. vespulae               AGATATTTGAACTTGGAATTGCTAGTAAATTTT-ATTAAATAAGTAGAATTGAATGTGTC
N. apis                   AGATATTTGAACTTGGAATTGCTAGTAAATTTT-ATTAAATAAGTAGAATTGAATGTGTC
E. hellem                 GGGCTTCTGAACGTGGAATTCCTAGTAAGAATG-ATTGAACAAGTTATTTTGAATGTGTC
S. intestinalis           GGGCTTCTGAACGTGGAATTCCTAGTAATAACG-ATTGAACAAGTTGTTTTGAATGGGTC
E. cuniculi               GGGCTTCTGAACGTGGAATTCCTAGTAATAGCG-GCTGACGAAGCTGCTTTGAATGTGTC
Pleistophora sp.          GGGCACACGAAAGAGGAATTCCTAGTAAGCGCC-CATCACCAGTGGGCGTTGAATCAGTC
E. schubergi              GGGCACACGAAAGAGGAATTCCTAGTAAGCGCC-CATCACCAGTGGGCGTTGAATCAGTC
N. corneum                GCACATACGAAAGAGGAATTCCTAGTAAGTGTG-TATCAACAATGGATATTGAATAAGTC
E. bieneusi               TACGTAGTGAATAAGGAATTCCTAGTAACGGTG-CCTCATCAAGGCATGGTGAATGTGTC
A. penaei                 TAAGTCATGAACTTGGAATTCCTAGTAATAATG-ATTCATCAAGTCATTGTGAATGTGTC
G. atherinae              AGGCTCAGGAACGAGGAATTGCTAGTAATCGCGGACTCATTAAGACGCGATGAATACGTC
Ichthyosporidium sp.      AGGCTCAGGAACGTGGAATTGCTAGTAATCGCGGACTCATTAAGACGCGATGAATACGTC
Vairimorpha sp.           TGCGTCATGAACGTGGAATTCCTAGTAGT-GGGCAGTCATTAACTGCACGCGAATGAGTC
A. michaelis              CG-CTCATGAACACGGAATAGCTAGTAA-CGTGAGTTCAATATACGGCGATGAATATGTC

                          1321                                                       1380
Thelohania sp.            ------------------------------------------------------------
T. solenopsae             ------------------------------------------------------------
N. bombycis               CCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGGTATTATCTATGA----ACAA
N. trichoplusia           CCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGGTATTATCTATGA----ACAA
V. necatrix               CCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGATATGTGTTGTGA----AATT
N. vespulae               CCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGATATATGTTGTGA----AATT
N. apis                   CCTGTTCTTTGTACACACCGCCCGTCGCTATCTAAGATGATATGTGTTGTGA----AATT
E. hellem                 CCTGTCCTTTGTACACACCGCCCGTCGCTATCTAAGATGAC----GCAGTGG----ACGA
S. intestinalis           CCTGTCCTTTGTACACACCGCCCGTCGCTATCTAAGATGAC----GCAGTGG----ACGA
E. cuniculi               CCTGTCCTTTGTACACACCGCCCGTCGCTATCTAAGATGAC----GCACTGGA---ACGA
Pleistophora sp.          CCTGTAGCTTGTACACACCGTCCGTCACTATCTCAGATG-T----TTTTCGGG---ATGA
E. schubergi              CCTGTAGCTTGTACACACCGTCCGTCACTATCTCAGATG-T----TTTTCGGG---ATGA
N. corneum                CCTGTAGCTTGTACACACCGCCCGTCACTATCTCAGATG-T----TTTTCAGG---ATGA
E. bieneusi               CCTGTTCTTTGTACACACCGCCCGTCACTATTTCAGATG-G----TCATAGGG---ATGA
A. penaei                 CCTGTAGCTTGTACACACCGCCCGTCACTGTCTCAGATG-G----TTGATGAG---ATG-
G. atherinae              CCTGTTCTTTGTACACACCGCCCGTCGTTATCGAAGATGGAGTCAGGCGCGA--ACAAG-
Ichthyosporidium sp.      CCTGTTCTTTGTACACACCGCCCGTCGTTATCGAATACGGTGCTCGGCGCGA--GCAAGG
Vairimorpha sp.           CCTGTTCTTTGTACACACCGCCCGTCGTTATCTAAGATGGA---AGTGCGGA--TGAGGT
A. michaelis              CCTGTTCTTTGTACACACCGCCCGTCGTTATCGAAGATGGAGTGATTTTTGAG-TCAATT

                          1381                                                       1440
Thelohania sp.            ------------------------------------------------------------
T. solenopsae             ------------------------------------------------------------
N. bombycis               ATTTATA---AAGTGAATAGATAGTACTAGATCTGATATAAGTCGTAACATGGTTGCTGT
N. trichoplusia           ATTTATA---AAGTGAATAGATAGTACTAGATCTGATATAAGTCGTAACATGGTTGCTGT
V. necatrix               AGTGAAAACTACTTGAACAATATGTATTAGATCTGATATAAGTCGTAACATGGTTGCTGT
N. vespulae               AGTGAAAACTACTTGAACAATATGTATTAGATCTGATATAAGTCGTAACATGGTTGCTGT
N. apis                   AGTGCAAGCTACTTGAACAATATGTATTAGATCTGATATAAGTCGTAACATGGTTGCTGT
E. hellem                 AGATTGAGAGGTCTGAGTCTTTCGTGTTAGATAAGATATAAGTCGTAACATGGCTGCTGT
S. intestinalis           AGATTGGAAGGTCTGAGTCCTTCGTGTTAGATAAGATATAAGTCGTAACATGGCTGCTGT
E. cuniculi               AGATCGGAAGGTCTGAGTCCTGAGTGTTAGATAAGATATAAGTCGTAACAAGGTAA----
Pleistophora sp.          AGAGTCTAGGCTCTGAATAACGGAAAGTAGATAAGATGTAAGTCGTAACATGGTTGCTGT
E. schubergi              AGAGTCCAGGCTCTGAATAACGGAAAGTAGATAAGATGTAAGTCGTAGCAAGGTTGCGGT
N. corneum                AGAGTCCAGGCTCTGAATAATGAAAAGTAGATAAGATGTAAGTCGTAACATGGTTGCTGT
E. bieneusi               AGAGCTTCGGCTCTGAATATCTATGGCTAGATAAAGTACAAGTCGTAACAAGGTTTCAGT
A. penaei                 ------------------------------------------------------------
G. atherinae              ---CGAGAGCGAGTGAGTGCAGGATTCTAGATGTGATACAAGTCGTAACATGGTTGCTGT
Ichthyosporidium sp.      TGAAATCACTGAGCGAGCGCAAGGTACCGGATCTGATACAAGTCGTAACAAGGTAGCTGT
Vairimorpha sp.           CGGTACGGCCGGACGAATCTGTGCTTGTAGATTGGATACAA-------------------
A. michaelis              ATAATTGGCTACTTGAATGAGTTATTCTAAAACCGGTACAAGTCGTAACAAGGCTACGGT
```

	1441 1466
***Thelohania* sp.**	-------------------------
T. solenopsae	-------------------------
N. bombycis	TGGAGAACCATTAGCAGGATCATAA
N. trichoplusia	TGGAGAACCATTAGCAGGATCATAA
V. necatrix	TGGAGAACCATTAGCAGGATCATAA
N. vespulae	TGGAGAACCATTAGCAGGATCATAA
N. apis	-------------------------
E. hellem	TGGAGAACCATTAGCAGGATCATAA
S. intestinalis	TGGAGAACCATTAGCAGGATCATAA
E. cuniculi	-------------------------
Pleistophora sp.	TGG----------------------
E. schubergi	CGGTGAACCATTAGCAGGATCATAA
N. corneum	TGG----------------------
E. bieneusi	TGGAGAACCATTAGCAGGATCATAA
A. penaei	-------------------------
G. atherinae	TGG----------------------
Ichthyosporidium sp.	AGGAGAACCATTAGCAGGATCATAA
***Vairimorpha* sp.**	-------------------------
A. michaelis	TGAAGAATCAGCAGTAGGATTAGCG

Figure 4.4. Multiple sequence alignment of the rRNA gene sequences of 19 species of microsporidia. Alignment of the sequences was done with the programs PileUp and CLUSTAL W (1.4). Names of microspridian species typed in bold indicate sequences obtained by the author. Dashes indicate gaps that were introduced to maintain alignments. Conserved regions are identified by '*'.

Sequence Data Analysis

For data analysis, about 70 bp were excluded at the 5' and 3' ends of the A. penaei sequence because of sequence uncertainties. The multiple sequence alignment (Figure 4.4) shows moderately variable regions in the 5'-end half and highly conserved stretches (denoted with '*') in the 3'-end half of the sequence. The distance matrix shown in Table 4.2 presents the mean distances between taxa, providing a comparison of the relative similarity between any two taxa. The ribosomal gene sequence of the protozoan G. lamblia (Sogin et al. 1991) was included as an outgroup. Mean distances and branching patterns of the phylogenetic tree (Figure 4.5) clearly showed that Thelohania sp. and T. solenopsae were very closely related (mean distance Thelohania sp./T. solenopsae = 0.008). A mean distance of 0.008 means 0.8 % sequence difference or 99.2 % sequence similarity. They were not closely related to any of the other microsporidia including the hymenopteran microsporidia N. apis and N. vespula (mean distance Thelohania sp./N. apis = 0.370, T. solenopsae/ N.apis = 0.376, Thelohania sp./N. vespula = 0.374, T. solenopsae /N. vespula = 0.378). They were also quite different from Vairimorpha sp. which can occur in dual infections together with Thelohania sp. in S. richteri (mean distance Thelohania sp./Vairimorpha sp. = 0.368, T. solenopsae/ Vairimorpha sp. = 0.367).The sequence of A. penaei, which was chosen as a close representative of the type species of the genus Thelohania, has a mean distance of 0.378 to Thelohania sp. and 0.366 to T. solenopsae. Vairimorpha sp. also was not closely related to any of the other microsporidia including V. necatrix, the type species of the genus Vairimorpha (mean distance Vairimorpha sp./V. necatrix = 0.366).

Based on the branching pattern of the phylogenetic tree (Figure 4.5), Vairimorpha sp. diverged first from the common ancestor, followed by the two Thelohania species which diverged after Vairimorpha sp. but before the other microsporidia included in the analysis. The phylogenetic tree also showed that Vairimorpha. sp. did not group with V. necatrix and the two Thelohania species did not group with A. penaei. In fact, Vairimorpha sp. and the two Thelohania species did not group closely with any of the other microsporidia. These findings were supported by the bootstrap analysis of the most parsimonious tree (Figure 4.6). A phylogenetic analysis of the other microsporidia to each other has been published (Baker et al. 1995).

Discussion

Molecular differences between species can be of great utility in diagnosing closely related forms, even where morphological or other traditional markers have failed or are ambiguous (Avise 1994). The results of the 16S rRNA gene sequence analyses indicated that Thelohania sp. and Vairimorpha sp. were two distinct species in two different genera. Furthermore, Thelohania sp. and T. solenopsae were the same species or two subspecies of the same species. Vairimorpha sp. did not belong into the genus Vairimorpha and the placement of the two Thelohania species and A. penaei into different genera is probably justified.

To draw meaningful conclusions based on comparative sequence analyses, guidelines to delineate different generea and species are needed. What percentage sequence similarity determines whether two species belong to the same genus or are the same species? Hartskeerl et al. (1993) proposed 16S rRNA gene sequence similarity levels of 70% and 90% respectively, to delineate species in different genera or the same genus. He also compared two isolates of E. bineusi-like microsporidia, believed to be different species based on site of collection (small intestine vs. maxillary sinus mucosa in humans), and found a 99% sequence similarity of the 16S rRNA genes. From these results he concluded that the two isolates are the same species. In the present study, 16S rDNA PCR product sizes, RFLPs, and sequence comparison of Thelohania sp. and Vairimorpha sp., the two microsporidia which may coinfect the same host, indicated that

Table 4.2. Pairwise distances between taxa

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	The. sp.	-	0.008	0.387	0.384	0.373	0.374	0.370	0.398	0.403	0.425	0.376	0.373	0.366	0.380	0.378	0.377	0.390	0.368	0.432	0.499
2	The. sol.	9	-	0.388	0.385	0.380	0.380	0.376	0.401	0.408	0.426	0.374	0.371	0.366	0.381	0.373	0.381	0.396	0.367	0.434	0.504
3	Nos. bom.	359	364	-	0.004	0.161	0.160	0.170	0.291	0.285	0.341	0.338	0.336	0.328	0.346	0.347	0.362	0.372	0.394	0.384	0.505
4	Nos. tri.	356	361	5	-	0.158	0.157	0.167	0.290	0.284	0.338	0.338	0.336	0.328	0.344	0.345	0.362	0.372	0.391	0.382	0.506
5	Vai. nec.	351	361	194	191	-	0.028	0.050	0.277	0.281	0.322	0.321	0.322	0.315	0.333	0.325	0.354	0.363	0.366	0.375	0.491
6	Nos. ves.	352	361	195	192	34	-	0.045	0.277	0.281	0.319	0.320	0.319	0.310	0.329	0.320	0.357	0.365	0.368	0.380	0.489
7	Nos. api.	345	354	198	195	59	54	-	0.291	0.293	0.328	0.324	0.329	0.318	0.339	0.328	0.366	0.382	0.368	0.382	0.496
8	Enc. hel.	396	403	345	344	331	332	338	-	0.089	0.152	0.313	0.311	0.314	0.317	0.352	0.370	0.372	0.373	0.418	0.458
9	Sep. int.	398	407	338	337	333	336	338	114	-	0.151	0.305	0.303	0.310	0.315	0.358	0.357	0.359	0.368	0.412	0.444
10	Enc. cun.	421	426	396	393	373	374	378	189	189	-	0.334	0.335	0.332	0.339	0.381	0.379	0.377	0.383	0.423	0.450
11	Ple. sp.	362	364	394	394	371	373	369	375	366	399	-	0.007	0.106	0.238	0.302	0.320	0.336	0.373	0.379	0.438
12	End. sch.	358	360	398	398	379	379	374	379	369	399	9	-	0.106	0.239	0.300	0.325	0.331	0.375	0.379	0.439
13	Nos. cor.	354	358	383	383	365	363	363	377	373	398	131	130	-	0.246	0.292	0.339	0.356	0.383	0.394	0.462
14	Ent. bie.	375	379	417	415	399	398	395	398	395	417	293	298	304	-	0.286	0.339	0.357	0.358	0.398	0.438
15	Agm. pen.	370	367	355	353	336	331	337	381	384	410	323	320	313	313	-	0.374	0.392	0.372	0.415	0.486
16	Glu. ath.	383	391	423	423	411	419	421	457	439	464	383	388	407	415	398	-	0.116	0.354	0.339	0.464
17	Ich. sp.	392	402	442	442	429	435	439	467	449	461	402	402	427	445	416	153	-	0.367	0.359	0.471
18	Vai. sp.	375	376	412	409	389	391	388	420	411	428	407	408	419	398	400	409	422	-	0.428	0.390
19	Ame. mic.	418	424	438	436	427	435	421	508	496	499	436	444	455	473	423	414	446	471	-	0.520
20	Gia. lam.	522	532	611	612	593	596	583	583	564	561	533	543	565	557	529	608	626	466	650	-

Species are as follows: 1, Thelohania sp.; 2, T. solenopsae; 3, Nosema bombycis (L39111*); 4, N. trichoplusiae (U09282); 5, Vairimorpha necatrix (Y00266); 6, N. vespula (U11074); 7, N. apis (X73894); 8, Encephalitozoon hellem (L19070); 9, Sepatata intestinalis (U09929); 10, E. cuniculi (Z19563); 11, Pleistophora sp. (U10342); 12, Endoreticulatus schubergi (L39109); 13, N. corneum (U11046); 14, Enterocytozoon bieneusi (L07123); 15, Agmasoma penaei; 16, Glugea atherinae (15987); 17, Ichthyosporidium sp. (L39110); 18 Vairimorpha sp.; 19, Ameson michaelis (L15741); 20, Giardia lamblia (M54878). Numbers above diagonal: mean distances (adjusted for missing data); numbers below diagonal: absolute distances.

* (GenBank accession number)

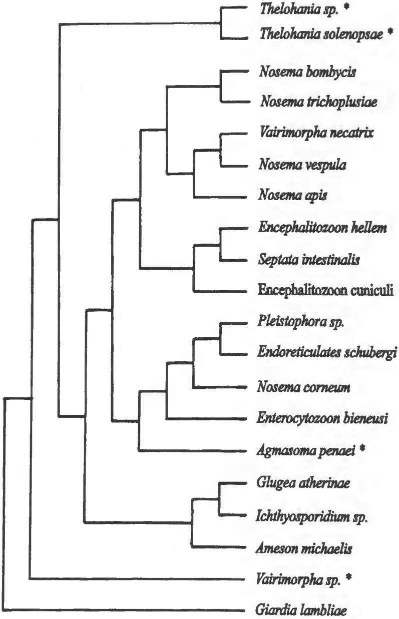

Figure 4.5. Phylogenetic tree (3,511 steps) based upon the 16S rDNA sequences of the 19 species of microsporidia aligned in Figure 4.4. Giardia lamblia was used as the outgroup. The tree, presented as a cladogram, was generated using the heuristic option of PAUP. Species, whose sequences were obtained by the author, are marked with '*'.

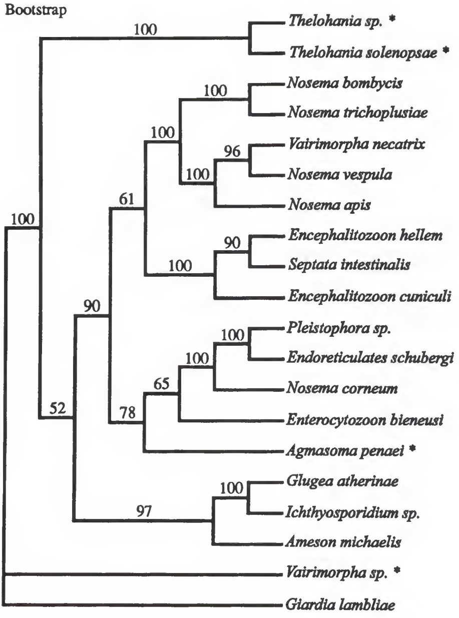

Figure 4.6. Bootstrap analysis (100 replicates) of the phylogenetic tree presented in Figure 4.5. Numbers on the tree indicate the percentage of bootstrap replicates which contained that topology. Bootstrap analysis was performed with PAUP. Species, whose sequences were obtained by the author, are marked with '*'.

their small subunit ribosomal gene sequences were very different. The PCR 1400 bp product of Thelohania sp. was roughly 100 bp larger than the PCR product of Vairimorpha sp. In addition, Vairimorpha sp. 16S rDNA had different restriction patterns than Thelohania sp. for several enzymes tested. Sequence comparison of the 16S rDNAs revealed a sequence similarity of 63.2% (or mean distance of 0.368) between the two microsporidia. These data, supported by Haartkeerl's et al. (1993) guideline of percentage sequence similarity for different genera, uphold the hypothesis that Thelohania sp. and Vairimorpha sp. represented two species in two different genera and not two phenotypes of the same species.

Even though spores of Vairimorpha sp. and Thelohania sp. were distinct at the light microscopic and ultrastructural level, they could have been different phenotypes of the same species. Many microsporidian species have more than one spore type such as the dimorphic genera Vairimorpha and Parathelohania (Sprague et al. 1992). Vairimorpha necatrix, for example, was initially described as two species, Nosema necatrix and Thelohania diazoma (Kramer 1965) because of its two morphologically distinct spore types. Later it was recognized that V. necatrix is a dimorphic species (Maddox 1966; Fowler and Reeves 1974; Pilley 1976). Another example of a polymorphic microsporidium is given by Becnel (1992) who described the heterosporous Amblyospora californica with three morphologically and functionally distinctive spore types. Vairimorpha necatrix, the type of the genus Vairimorpha, shared ~ 63% sequence similarity with Vairimorpha sp. which was indicative that Vairimorpha sp. may not belong in the genus Vairimorpha. Other data such as ultrastructure of the spores support this hypothesis. Both spore types of Vairimorpha sp. are ultrastructurally distinct from V. necatrix. For example, free spores of V. necatrix (Mitchell and Cali 1993) and Vairimorpha sp. differ in the arrangement of the polar filament and polaroplast structure, and meiospores of the two species differ in thickness of exospore and endospore. Different sized 16S rDNA PCR products and a sequence similarity of ~63% of the

Thelohania species with *A. penaei* also indicated that *A. penaei* and the two *Thelohania* species belonged different genera. The sequence of *A. penaei* is presently the only sequence available of a microsporidium close to *Thelohania*. Generally, other studies also agree with Hartskeerl's et al. (1993) suggestion of the 70% and 90% cutoff for different genera and different species within the same genus. For example, *E. cuniculi* shares about 72% sequence similarity with *V. necatrix* (Schuitema et al. 1993). Vossbrinck et al. (1993) find a 90% sequence similarity between *V. necatrix* and *V. lymantriae* and 77% sequence similarity between *E. cuniculi* and *E. hellem* in regions of the small and large subunit rDNA and the internal spacer. These data, even though quite different from each other, fall within the 70-90% range for different species within the same genus.

On the other hand, sequence similarities of *S. intestinalis* and *E. cuniculi* or *E. hellem* are 77% and 73% respectively (Weiss et a. 1994), which according to Weiss et al. (1994) supports placement of these species into different genera which would not be in accordance with Hartskeerl's et al. (1993) proposed rule. However, Hartskeerl et al. (1993) and Baker et al. (1995) suggested to reclassify *S. intestinalis* as *E. intestinalis* based on reanalysis of the 16S rDNA sequences. They find sequence similarities of about 90% (Hartskeerl et al. 1993) and 94% (Baker et al. 1995) between *S. intestinalis* and both species of *E. hellem* and *E. cuniculi* (Note: The mean distances for *S. intestinalis*/*E. hellem* and *S. intestinalis*/*E. cuniculi* (Table 4.2) are 0.089 and 0.151 (that is *S. intestinalis*/*E. hellem* and *S. intestinalis*/*E. cuniculi* have 91% and 85% sequence similarities). The slight discrepancy of the results reported in this study and the results published previously is probably because not exactly the same regions of the 16S rDNA were aligned and analyzed).

Looking at the available data, one must keep in mind that molecular taxonomy is most convincing when supported concordantly by multiple lines of evidence (Avise 1994). Placement of microsporidia into different genera or species based on a certain percentage sequence similarity is subjective and, because of lack of clear-cut sexual stages, should be supported by inclusion of other available characters such as morphology, host and tissue specificity, biochemical profiles and available classification schemes.

PCR product size and RFLPs of Thelohania sp. and T. solenopsae were identical for the enzymes tested, and sequence comparison showed 99.2% identity (or a mean distance of 0.008) between the two microsporidia. According to Hartskeerl et al. (1993), the high sequence similarity would place them in the same species. The sequence analyses data in conjunction with light microscopic and ultrastructural studies of the spore morphology indeed support the hypothesis that Thelohania sp. and T. solenopsae may be the same species or two closely related subspecies. Similarly, Pleistophora sp. and E. schubergi, two microsporidia infecting Lepidoptera, have a very small 16S rDNA sequence difference (mean distance = 0.007), and Pleistophora sp. should be reclassified as an Endoreticulatus sp. (Baker et al. 1995). Joseph Maddox suggests (personal communication) to consider Pleistophora sp. and E. schubergi as intraspecific variants of one species, E. schubergi, because they are indistinguishable based on tissue specificity (both are midgut parasites), ultrastructure and almost identical 16S rDNA sequences.

In contrast, E. hellem and E. cuniculi, also indistinguishable by ultrastructure and development, have a 16S rDNA sequence mean distance of 0.152 (Table 4.2). It should be noted that Baker et al. (1995) published a mean distance of 0.066 between E. hellem and E. cuniculi. This difference is due to the fact, that Baker et al. (1995) excluded parts of the 16S rDNA sequences in their analysis. They analyzed only those characters which they could align unambiguously. The mean distance of 0.152 supports the classification of E. hellem and E. cuniculi as two separate species (even though they are indistinguishable by fine strucure and development) as shown by Didier et al. (1993) with immunological and biochemical tests.

The two respective host ants of T. solenopsae and Thelohania sp., S. invicta and S. richteri, are also very closely related, and it is still debated whether they represent color morphs of one species or two separate species (Vander Meer and Lofgren 1988). To make a final determination on whether T. solenopsae and Thelohania sp. are conspecific, crucial data on the life cycles and host specificities of these microsporidia are still needed. For example, can Thelohania sp. infect S. invicta and T. solenopsae infect S. richteri (cross-infectivity), and can the infection cycle be completed successfully? As trivial a question this may seem, so far it is not even possible to infect S. invicta with T. solenopsae and S. richteri with Thelohania sp. under laboratory or field conditions (data not shown; R.S. Patterson personal communication).

The following example illustrates why it is important to have data on cross-infectivity under natural conditions with the two Thelohania species in support of the sequence analysis data. Nosema bombycis and N. trichoplusiae have almost identical 16S rDNA sequences (mean distance = 0.004). They belong to a group of indistinguishable Nosema species infecting Lepidoptera (Nordin and Maddox 1974). However, even though N. trichoplusiae and N. bombycis have very similar life cycles, ultrastructural characteristics, and 16S rDNA sequences, J. Maddox (personal communication) proposes to treat them as two different species because cross-infectivity (possible in the laboratory) between their hosts has not been demonstrated under natural conditions.

When comparing closely related sequences, one must also be aware of possible sources of variation which include (1) error rate of DNA polymerase during PCR and sequencing reaction (Barnes 1994), (2) variation among the different copies of the 16S rRNA gene, and (3) intraspecific variation (Li and Graur 1991). The error rate (or number of mutations per base per cycle) of *Taq* DNA polymerase is about $25\mathrm{x}10^{-6}$ (Boehringer Mannheim Biochemica Bulletin No. 3 1995). The error frequency of Primezyme$^{\mathrm{TM}}$ DNA polymerase is less than $80\mathrm{x}10^{-4}$ (Biometra Catalog 1994). This fact is not important when sequence differences are big, but it is more important when sequence differences are very small. To compensate for fidelity problems of the DNA polymerases, both strands were sequenced at least three times.

Even though multiple copies of nuclear rRNA genes do not evolve independently but in concert (Arnheim 1983) there may be a low level of heterogeneity at about 0.1% of the nucleotide positions among rDNA within individuals and throughout species (Mylvaganam and Dennis 1992). Again, this low level of heterogeneity is not important when comparing dissimilar sequences but gains importance in the comparison of very closely related sequences. Since PCR products of Thelohania sp., Vairimorpha sp. and A. penaei were sequenced directly, no conclusions about intraindividual or intraspecific variation can be drawn. Sequence data of three clones of the 16S rRNA gene of T. solenopsae showed a very low level of heterogeneity (about 0.1%), but this is not reflective of intraindividual or intraspecific variation. Cloning of the PCR product selects single molecules which may harbor nucleotide misincorporations due to the error rate of the DNA polymerase. Intraindividual and intraspecific variation of microsporidia need to be investigated in further studies. Intraindividual variation of the multiple copies of the 16S rRNA gene would require cloning of the PCR product of single spore isolates. In an initial study to research intraspecific variation of fire ant microsporidia, 16S rDNA sequences of the same microsporidian species, Thelohania sp., isolated from different S. richteri ant colonies, could be compared. The different isolates could be regarded as different 'individuals' of the same species. The advantage of working with Thelohania sp. instead of Vairimorpha sp. or T. solenopsae is that we have plenty of material available of Thelohania sp.but not the other species.

Yet another consideration is that two species may still not be the same species even if they have an identical 16S rDNA sequence (which does represent but a small portion of the genome) (J. J. Becnel, personal communication). It is prudent to confirm results found by sequence comparison of one gene with sequence comparisons of another gene. Studies on sequence comparisons of another gene such as the 23S rRNA gene or internal spacer (Vossbrinck et al. 1993) to corroborate results from sequence comparisons of the 16S rDNA gene should be initiated. The cytochrome *c* gene (Woese 1987) which is frequently used in eukaryotes, may also be present in microsporidia and could be used for additional sequence analyses.

The phylogenetic tree (Figure 4.5) in this study is similar to the tree published by Baker et al. (1995) except that in our analysis, V. oncoperae and a different Vairimorpha sp., used by Baker, were not included. In addition to the species whose sequences were obtained by the author, we also included G.atherinae in the analysis. Baker et al. (1995) found four groups: The Ichthyosporidium group (comprised of A. michaelis, Vavraia oncoperae and Ichthyosporidium sp.), the Encephalitozoon group (comprised of E. hellem, E. cuniculi and S. intestinalis), the Vairimorpha/Nosema group (comprised of N. apis, N. vespula (also called Nosema sp.), N. trichoplusiae, N. bombycis, V. necatrix , and Vairimorpha sp.) and the Endoreticulates group (E. schubergi, E. bieneusi, N. corneum and Pleistophora sp.). The Vairimorpha sp. in Baker's study is not the same Vairimorpha sp. used in this study. It was isolated and identified from the gypsy moth Lymantria dispar by J. Maddox while the one in the present study was found in the black imported fire ant S. richteri.

We obtained the same groups in our phylogenetic tree. Glugea atherinae was placed in the Ichthyosporidium group (bootstrap value of 97%). Agmasoma penaei was placed in the Endoreticulates group (bootstrap value of 78%). In addition, we found two new taxon groups, one comprised of the two Thelohania species (bootstrap value of 100%) and one comprised of Vairimorpha sp. More species need to be analysed however, to support the validity of these two groups. The tree branching pattern (Figure 4.5) and the mean distance between Vairimorpha sp. and V. necatrix of 0.366 showed that Vairimorpha sp. is unrelated to the 'true' Vairimorpha.

The study of Baker et al. (1995) also supports the need to rearrange the current microsporidian classification. They determined, based on mean distance and branching pattern of the phylogenetic tree, that N. corneum is not a true Nosema species (Figure 4.5) but more closely related to E. schubergi.. They reported that the 16S rRNA gene sequence of *N. corneum* has only 72% sequence similarity with *N. bombycis*. It has a 93% sequence similarity with *E. schubergi*.

In conclusion, the sequence analyses data in conjuction with other information such as ultrastructure and tissue specificity of the fire ant microsporidia support the hypotheses that (1) *Thelohania* sp. and *Vairimorpha* sp. are two distinct species in two different genera and not mere phenotypes of the same species, (2) *Thelohania* sp. and *T. solenopsae* are the same species or two subspecies of the same species, (3) *Vairimorpha* sp. does not belong into the genus *Vairimorpha* and (4) *A. penaei* and the two *Thelohania* species are separate genera. Data on cross-infectivity of *Thelohania* sp. and *T. solenopsae* in their respective hosts are needed however, to draw a biologically meaningful final conclusion.

CHAPTER V
SUMMARY AND DIRECTION OF FUTURE RESEARCH

Synopsis

Comparative phenotypic (light microscopic and ultrastructural features) and genotypic (sequence comparison of the 16S rRNA genes) analyses of the fire ant microsporidia support the following conclusions:

(1) Vairimorpha sp. and Thelohania sp. are distinct species in separate genera and not mere different phenotypes of the same species. (2) Thelohania sp. and T. solenopsae are either two subspecies of the same species or conspecific. Cross-infectivity in field situations needs to be demonstrated, however, to make this conclusion biologically meaningful. In other words, infection and completion of the infection cycle of S. invicta with Thelohania sp. from S. richteri, and of S. richteri with T. solenopsae from S. invicta must be achieved. (3) Vairimorpha sp. does not belong in the genus Vairimorpha.

Additional evidence to corroborate conspecificity could focus on comparative sequence analysis of another gene (perhaps the 23S rRNA gene or the cytochrome *c* gene). Since microsporidia do not have mitochondria and virtually nothing is known about their metabolic pathways, the presence of a cytochrome *c* gene must first be demonstrated.

Qualitative and quantitative FAME profile differences were detected in the different microsporidian species but the host insect did influence the FAME profile of a microsporidium. Improved methodology could render FAME analysis as a useful taxonomic character. Future experiments could include standardization of microsporidian growth through the development of *in vitro* culture techniques. Furthermore, the sample size presently required for FAME analysis (1×10^9 spores) could be reduced by scaling down the extraction and derivatization procedure. Also, FAME profiles of a wide variety of microsporidian genera and species should be determined to look for signature fatty acids that would be present only in one genus (or species).

APPENDIX

Cuticular hydrocarbon analysis of Solenopsis richteri and S. invicta

1. Soak five worker ants, frozen at -20°C until analysis, in 1 mL of hexane in small vials for 2 h to extract lipids.
2. Separate hydrocarbons from the extracted lipids with chromatography on mini-columns containing 3 cm of silica gel (60-200 mesh, J.T. Baker, Philadelphia, Pa.) packed into disposable Pasteur pipettes (Carlson and Bolten 1984). To make a column, stuff a little glass wool into a Pasteur pipette and pour silica gel on top of it.
3. Elute hydrocarbons from the mini-column with 3 mL of hexane, concentrate them to dryness with nitrogen gas, and redissolve them in 20 µL of hexane for GC analysis (Carlson and Brenner 1988).
4. Analyze hydrocarbons with a 5890 series II Hewlett Packard gas chromatograph with flame ionization detector fitted with a 30 m x 032 mm i.d. x 0.25 µm film thickness fused silica capillary column of DB-1.
5. Following a cool-on column injection of 1 µL at 63°C, raise oven temperature to 230°C at 25°C/min, and then to a final temperature of 320°C at 7°C/min. Hold temperature at 320°C for 15 min. The carrier gas was hydrogen.
6. Process data by HP Chemstation, version 1.0 software.

Per os infection of corn earworm, Helicoverpa zea with Vairimorpha necatrix

1. Sprinkle corn earworm eggs on pinto bean diet in 50 well flats, keep them in the insectary at 23°C with a photo period of 16 h light/8 h dark. Secure flats with clear plastic foil and solid metal or plastic sheet to prevent larvae from escaping.
2. After approximately 5 days, discard all but one healthy larva from each well. Add 10 µL of 1x10^{6} spores/mL to the diet of each larva.
3. Harvest the spores from last-instar larvae.

Per os infection of H. zea with Nosema algerae

1. Sprinkle corn earworm eggs on pinto bean diet in 50 well flats, keep them in the insectary at 23°C with a photo period of 16 h light/8 h dark.
2. Pick 4-5 day old H. zea larvae and starve them individually in small plastic cups for 24 h.
3. After 24 h, add 20 µl of 1x10^{7} spores/mL deionized water to each cup.
4. Expose the larvae for 24 h to the spore suspension.

5. Place larvae on pinto bean diet (one larva/well); secure flat with clear plastic foil and solid sheet of metal or plastic to prevent larvae from escaping.
6. Harvest the spores from adult moths.

Per os infection of Anopheles quadrimaculatus with N. algerae

1. Sprinkle 1000 mosquito eggs onto 100 mL infusion water and keep in insectary at 23°C with a photoperiod of 16 h light/8 h dark. Infusion water consists of a 0.015% powdered liver:brewer's yeast (1:1) suspension.
2. After 24 h, make slurry with ground alfalfa; pour into 150 mL container. Add 1×10^5 spores in aqueous suspension and the neonate larvae in 100 mL hatching water. The final concentration of the alfalfa suspension should be 0.03 %.
3. After 24 h, make alfalfa slurry for 3 L water. Pour slurry into big rearing pan, add mosquito larvae and 3 L of deionized water. The final concentration of the alfalfa suspension should be 0.03%.
4. Check after 48 h if larvae need to be fed (1 g of powdered liver:brewer's yeast (1:1) in slurry).
5. After additional 48-72 h feed larvae with 2 g of powdered liver:brewer's yeast:hog chow (40% protein) (1:1:1) mix in slurry.
6. Feed again after 24-48 h with 1 g of powdered liver:brewer's yeast:hog chow (40% protein) (1:1:1) mix slurry.
7. When larvae start pupating pick pupae daily into small cups and keep in mesh cages until emergence. Add cotton balls saturated with 10 % dextrose to maintain adults.
8. Harvest the spores from the adult mosquitoes 3-5 days post-emergence.

Purification of V. necatrix from H. zea

1. Establish continuous density gradient with Ludox (a colloid of 40% silica in NaOH solution, pH 9.8 and specific gravity ρ of 1.303) using gradient mixer. Load 15 mL of Ludox in one chamber and 15 mL of deionized H_2O in the other chamber; a magnetic stirrer in the Ludox chamber mixes the diluent with the Ludox. A plastic hose drains the Ludox chamber into a 30 mL centrifuge tube (Undeen and Alger 1971).
2. Surface-sterilize last-instar infected larvae in 70% ethanol and cut open in a dissecting dish.
3. Remove fat bodies without lacerating gut tissues, place in deionized water and grind in glass tissue grinder.
4. Strain resulting suspension through cotton plug in glass syringe to remove large body parts.
5. Centrifuge at 4,080 g in swinging-bucket head rotor (Sorvall S 34 centrifuge) for 15 min.
6. Discard supernatant and wash pellet once in deionized H_2O. Resuspend pellet in ~ 500 µL of deionized H_2O.
7. Layer suspension on continuous Ludox gradient.

8. Centrifuge at 16,320 g for 30 min in swinging-bucket head rotor.
9. Draw off the bands with Pasteur pipette, examine samples under microscope to confirm identity of purified organisms.
10. Wash spores twice in deionized H_2O to remove Ludox. Store in deionized H_2O at 4°C.

Purification of N. algerae from H. zea

1. Establish continuous density gradient with Ludox (a colloid of 40% silica in NaOH solution, pH 9.8 and specific gravity ρ of 1.303) using gradient mixer.
2. Rinse adult infected moths in water, clip off wings, and triturate in Tekmar Tissumizer in deionized water.
3. Strain through cotton plug in glass syringe to remove large body parts.
4. Follow steps 5-10 of V. necatrix purification protocol.

Purification of N. algerae from A. quadrimaculatus

1. Establish continuous density gradient with Ludox (a colloid of 40% silica in NaOH solution, pH 9.8 and specific gravity ρ of 1.303) using gradient mixer.
2. Immobilize adult infected mosquitoes by chilling them at -20°C for about 3 min. Aspirate them with an aspirator connected to a vacuum pump.
3. Homogenize the mosquitoes with a small amount of deionized water in a Waring blender.
4. Strain resulting suspension through cotton plug in glass syringe to remove large body parts.
5. Follow steps 5-10 of V. necatrix purification protocol.

Purification of Thelohania sp. from S. richteri

1. Establish continuous density gradient with Ludox (a colloid of 40% silica in NaOH solution, pH 9.8 and specific gravity ρ of 1.303) using gradient mixer.
2. Homogenize infected ants with Tekmar Tissumizer in ant homogenizing buffer (0.1% SDS, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA).
3. Strain through cotton plug to remove large body parts.
4. Centrifuge at 4,080 g in swinging-bucket head rotor (Sorvall S 34 centrifuge) for 15 min.
5. Discard supernatant and wash pellet once in deionized H_2O.
6. Incubate pellet in 10 µg/mL proteinase K and 1/4 volume of pansporoblastic membrane disruption buffer (4% SDS, 25 mM EDTA, 50 mM Tris-HCl, pH 7.5) for 10 min at 40°C.
7. Follow steps 7-10 of V. necatrix purification protocol.

Fatty acid methyl ester (FAME) extraction of microsporidia

1. Pipette $1x10^9$ spores into a 13x100 mm culture tube and store overnight at 4°C to allow the spores to settle.
2. The next day, carefully withdraw the supernatant. Add 1 mL of 15% NaOH in 50% methanol, seal tube, and saponify fatty acids at 100°C for 30 min.
3. Upon cooling, add 2 mL of 6 N HCl in 50% MeOH, recap tube, heat at 80°C for 10 min to methylate the fatty acids.
4. Solvent-extract fatty acid methyl esters (FAME) from the aqueous phase with 1.25 mL of hexane:methyl-tert-butyl ether (1:1; v/v).
5. Wash organic phase with 3 mL of 1.2% aqueous NaOH and transfer to a gas chromatograph (GC) vial.

Fatty acid methyl ester (FAME) analysis with gas chromatography (GC)

1. Analyze FAME with a Hewlett Packard 5890 gas-liquid chromatograph fitted with an Ultra 2 fused silica capillary column (25 m x 0.2 mm i.d. x 0.33 µm film thickness) coated with 5% phenyl methyl silicone.
2. Inject 1 µL of FAME sample. Raise temperature from 170^{o}C to 270^{o}C in 5^{o}C/min increments using hydrogen as the carrier gas. After flame-ionization, measure FAME peaks by a Hewlett Packard 3392 integrator and express as percentages of the total FAME profiles.

Analysis of FAME mixtures by coupled gas chromatography - mass spectrometry (GC-MS)

1. Analyze aliquots of the FAME mixtures on a Perkin Elmer 8420 GC interfaced with a Finnigan Ion Trap Detector (ITD, Model 6210), with INCOS data collection software and a 80286 computer. The GC-MS was fitted with a 25 m x 0.25 mm i.d. DB-1 fused silica capillary column.
2. Inject 1 µL samples in a splitless mode followed with a purge flow of helium after 30 sec. The carrier gas was helium with a flow rate of 25 cm/sec. The initial temperature of the column was 60°C, and following injection the temperature was programmed to 150°C at 30°C/min, then programmed to 220°C at 5°C/min, and held for a total running time of 100 min.

Transmission electron microscopy

Fixation:

1. Dissect specimen in 2.5% glutaraldhyde.
   After 5 minutes or so, specimen can be cut into smaller pieces.
2. Transfer pieces to fresh glutaraldhyde and fix for total of 2.5 hours.

3. Wash in 0.1 M cacodylate buffer (pH 7.2 - 7.3) 3 times at 15 minutes each (for a total of 45 minutes).
4. Postfix in 1.0% osmium tetroxide (pH 7.5) for 1 hour 45 min. to 2 hours.
5. Double distilled washes - 3 times at 15 minutes each (for a total of 45 minutes).
6. Begin dehydration or for extended storage use sucrose buffer.

Dehydration:

1. 10% ETOH - 10 minutes
2. 30% ETOH - 10 minutes
3. 50% ETOH - 10 minutes
4. 70% ETOH - 10 minutes
5. 80% ETOH - 10 minutes
6. 90% ETOH - 10 minutes
7. 95% ETOH - 10 minutes
8. 100% ETOH - 15 minutes
9. 100% ETOH - 15 minutes
10. 100% Acetone - 15 minutes
11. 100% Acetone - 15 minutes

Put specimen into plastic dilutions.

Infiltration and Embedding:

1. 25% resin: 75% absolute acetone - overnight
2. 50% resin: 50% absolute acetone - 4 hours
3. 75% resin: 25% absolute acetone - 4 hours
4. Pure resin overnight
5. Pure resin (change vials) - all day (»6 hours)*

   *For better infiltration, especially for spores, extend the specimen in pure resin for another day (overnight) or over the weekend. Embed as usual.
6. Embed in Beem capsules which have dried at least 24 hours in a 60°C oven. Make sure to include label with block number when embedding. Leave in oven overnight.
7. Remove the embedded blocks next morning and let them cool off before cutting out blocks.
8. Blocks are now ready for thick-and thin-sectioning.
9. Stain thin section with methanolic uranyl acetate (50% methanol, 1% uranyl acetate) for five min. Rinse grids in deionized H2O, blot dry and stain with lead citrate (Reynolds 1963) for 5 min. To stain, immerse the grids into droplets of lead citrate. Blot dry.

PCR protocol

1. For one 25 μL reaction, prepare master mix in a sterile, 0.5 mL microfuge tube. To prevent carry-over contamination, use plugged cotton tips.

10x buffer*	2.50 μL
10x nucleotide mix (2 mM dATP/dGTP/dTTP/dCTP	2.50 μL
primer 1 (4 pmol/μL)	1.00 μL
primer 2 (4 pmol/μL)	1.00 μL
sterile distilled (sd) H_2O	7.84 μL
DNA *Taq*Pol 0.8 U/reaction	0.16 μL
or Primezyme^{TM} DNA pol. 0.3 U/reaction	0.16 μL

**Taq*Pol 10x reaction buffer: 100 mM Tris-HCl, 500 mM KCl, and 25 mM $MgCl_2$. Primezyme^{TM} DNA polymerase 10x reaction buffer: 100 mM Tris-HCl, 500 mM KCl, 1% Triton X-100, and 25 mM $MgCl_2$

Add the enzyme after the master mix has been heated for 5 min.
Final volume of master mix is 15 μL. Keep the master mix on ice.

2. To another sterile 0.5 mL microfuge tube, add DNA template in a volume of 10 μL. Overlay with either 100 μL sterile glycerol or 50 μL Chill-out 14^{TM} Liquid Wax (MJ Research). Heat template and master mix in heating block to 94°C for 5 min. Add DNA polymerase to master mix and vortex. Add 15 μL of master mix to template. Start temperature cycling (1 min at 94°C, 1 min at 52°C, 1 min at 72°C for 35 cycles followed by a final extension step of 72°C for 15 min).
3. Mix 5 μL aliquot of PCR product with 5 μL of 1x loading dye (10x loading dye: 50% glycerol, 50 mM EDTA, 0.5% bromophenol blue) and electrophorese on a 0.8% Seakem LE agarose gel in 1x Tris-acetate running buffer (TAE; 40 mM Tris-acetate, pH 8.0, 1 mM EDTA, pH 8.0).
4. Purify PCR products with the QIAquick PCR Purification Kit (QIAGEN), elute in sdH_2O or TE (10 mM Tris-HCl, pH 8.0, 4 mM EDTA, pH 8.0) and store at -20°C.

   QIAquick PCR purification:

   Add 5 vol (e.g. 500 μL) of buffer PB to 1 vol (e.g. 100 μL) of PCR reaction and mix. Place a QIAquick spin column into a 2 mL collection tube and load the sample. Centrifuge 30-60 sec at maximum speed. Drain flowthrough fraction from collection tube and place QIAquick column back in the same tube. To wash, add 750 μL of buffer PE to column and centrifuge 30-60 sec. Drain buffer PE flowthrough from existing tube and spin column again to remove residual buffer PE. Place column in a clean 1.5 mL microfuge tube. To elute DNA, add 50 μL of 10 mM Tris-HCl, pH 8.0 or 50 μL of sdH_2O to column and centrifuge for 30-60 sec.

Cloning of PCR product

1. Clean PCR product (has restriction sites added to ends) on Qiaquick PCR purification column. Use 100 μL of crude PCR product. Elute bound DNA from column with 50 μL of TE (10 mM Tris-HCl, pH 8.0, 4 mM EDTA, pH 8.0).

2 Load aliquot on gel to check recovery rate.

3. Add equal amount (= 50 µL) of 20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS to column-purified PCR product.

4. Add proteinase K (stock of 5 mg/mL) to final concentration of 5 µg/100 µL. Incubate at 37°C for 30 min.

5. Clean on Qiagen column, load aliquot to check recovery rate.

6. Double-digest with the appropriate restriction enzymes (New England Biolabs) to create overhanging ends.

   48.0 µL DNA in TE, pH 8.0
   1.0 µL of each restriction enzyme
   5.5 µL of 10x restriction buffer (New England Biolabs)
   Incubate at 37°C for 4 h.

   For restriction enzyme digest of PCR products use *Bam*HI (20 U/µL) and *Kpn*I (15 U/µL) to digest Vairimorpha PCR products (sequential digest because of different buffer -low salt followed by high salt- requirements), use *Bam*HI (20 U/µL) and *Eco*RI (20 U/µL) to digest Thelohania PCR products (double digest).
   Note: Double-digest the plasmid DNA (pTZ 19R) at the same time and purify (can use Qiagen column or SeaPlaque agarose purification).

7. Clean double-digested DNA on Qiagen column and load aliquot on gel to check recovery and digestion.

8. Controls to see if digestion worked:
   Electrophorese 1.0 µL aliquot of 100 ng/µL uncut plasmid
   Electrophorese 2.0 µL aliquots of plasmid digested with either enzyme. Include 1 kb bacteriophage lambda (λ) DNA ladder (BRL Life Technologies. Inc.).

9. Ligation of PCR product to plasmid DNA:

   17.0 µL DNA suspension
   2.0 µL 10x ligation buffer
   1.0 µL T4 ligase (400 U/µL) (New England Biolabs)

   Note: Use a 1:3 mole ratio of vector (pTZ 19R) to PCR product in ligation mix.
   For example: If using 200 ng DNA of a 2.9 kb plasmid, how much PCR product (1.2 kb) DNA should be used? 200 ng plasmid DNA/2.9 kb = ? ng PCR product DNA/1.2 kb x 3
   Ligate at room temperature for 4 h in the dark; then store at 4°C.
   Control: Ligate digested PCR product DNA to each other (not into plasmid). Run gel; expect to see ladder of bands if digestion and ligation worked well; will see only primer-dimers if digestion and/or ligation didn't work.

10. Transformation of E. coli JM109 competent cells (have the F episome which allows them to be infected by M13 to produce single-stranded DNA):
   Heat the ligation mix at 65°C for 10 min to inactivate enzyme and enhance transformation
   Put ice bucket with ice into the hood.
   Remove 50 µl aliquots of competent cells from -70°C freezer; thaw on ice.
   Add 1 and 5 µl of DNA ligation reaction directly to the cells, moving the pipette through cells while dispensing.
   Incubate cells on ice for 30 min.

Incubate cells for 30 sec. at 37°C; do not shake.

Place on ice for 2 min.

Add 0.95 mL of room temperature S.O.C. medium and mix gently.

S.O.C. medium (per 1 liter diH_2O): 2% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), 0.05% NaCl (w/v), 2.5 mM KCl; adjust to pH 7.0 with 5 N NaOH, autoclave, let cool to ~ 60°C and add 20 mL of sterile solution of 1 M glucose (20 mM glucose final conc.); just before use, add 5 mL of sterile solution of 2 M $MgCl_2$.(10 mM $MgCl_2$ final conc.).

Grow cells at 225 rpm and 37°C for 1 h.

Spread 100-200 µl of the cell suspension on LB plates.

LB media: 1% bacto-tryptone (w/v), 0.5% bacto-yeast extract (w/v), 1% NaCl (w/v).

10 LB plates: Autoclave 100 mL of LB media + 1.5 g agar (bacto) in a 500 mL flask. When still pretty warm, add 100 µL of X-Gal (20 mg/mL stock) which gives a final conc. of 20 µg/mL and 4 mL of ampicillin (2.5 mg/mL stock) which gives a final conc. of 100 µg/mL of ampicillin. Then pour ten plates.

Keep remaining cell suspension at 4°C overnight.

If no colonies grew on plate after one night, concentrate cells of remaining cell suspension by centrifugation for 5 sec. Resuspend in 100 µl of medium.

Remove 10- and 1- µl aliquots and add 100 µl of medium to them. Plate out these dilutions and the remaining 90 µl.

Incubate overnight at 37°C. The ampicillin-resistant white colonies carry the plasmid with ligated DNA.

Controls: Plate out cells transformed with (a) uncut plasmid, (b) double-digested plasmid, (c) double-digested plasmid ligated to PCR product DNA.

E. coli colony hybridization: dot blots

Day 1

1. Plate E. coli transformants from LB plates (amp^+, X-gal^+) onto LB plates (amp^+) with numbered grid lines. Incubate over night at 37°C for 16 h.

Day 2

1. Use a soft pencil and spot a Hybond-N nylon membrane 1 cm apart, 10 spots per row. Resuspend a toothpick head full of E. coli transformants in 10 µL LB broth and spot 1µL of cell suspension onto the markings of the membrane.
2. Immerse the membrane in 0.5 M NaOH/1.5 M NaCl solution for 30 sec. Make sure the membrane is completely immersed in the solution.
3. Transfer the membrane to 0.5 M Tris-HCl, pH 8.0/1.5 M NaCl solution and immerse for 5 min.
4. Transfer membrane to 6x SSC and immerse for 5 min.

20x SSC: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H_2O. Adjust to pH 7.0 with 10 N HCl. Aliquot and autoclave.

5. Wrap the membrane in Whatman blot paper and bake at 80°C for 2 h under vacuum. Store the membrane in drawer.

Day 3

1. Place membrane in hybridization tube and wet it in 5 mL of 6x SSC. Discard 6x SSC.
2. Add prehybridization solution at 10 mL per 10 cm^2 of membrane. Rotate in hybridization chamber for 4-6 h at 68°C.
   Prehybridization/Hybridization solution (100 mL)
   - 200 µL EDTA (0.5 M)
   - 35 mL of 20% SDS
   - 50 mL Na_2HPO_4 pH 7 (1 M)
   - 15 mL H_2O
3. Nick translate PCR product DNA.

PCR product DNA (~40 ng/µL)	3 µL
TE	10 µL
10x Nick buffer*	3 µL
100 µM dNTP	1 µL each
^{32}P-dCTP-3000	5 µL (50 µCi)
DNAase	3 µL (1 mg/mL)
DNA Pol I (5U/µL)	3 µL

*0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 µg bovine serum albumine (BSA).

Incubate at 15°C for 45 min. Mix the probe with 1 mL TE and pass over a Sephadex-50 packed column. Reject the first 0.5 mL flowthrough and collect the rest in a sterile glass tube. Add 2 mL TE to the column after the probe got into the column and collect the flowthrough. Boil the probe by placing the glass tube in a boiling water bath for 3 min and mix with ~ 5 mL hybridization solution before adding to the membrane.

4. Remove prehybridization buffer and hybridize the membrane in 10 mL hybridization buffer containing the radioactive probe for 16 h at 68°C.

Day 4

1. Wash the membrane with washing solution I 2 times in ~50 mL fluid at 68°C for 1 h each. Then wash membrane wish washing solution II 2 times in ~50 mL fluid at 68°C for 1 h each.
   Washing solution I (5% SDS conc.; per liter)
   - 2 mL EDTA (0.5 M)
   - 40 mL Na_2PO_4 pH 7.2 (1 M)
   - 250 mL 20% SDS
   - 708 mL H_2O

   Washing solution II (1% SDS; per liter)
   - 2 mL EDTA (0.5 M)
   - 40 mL Na_2PO_4 pH 7.2 (1 M)
   - 50 mL 20% SDS
   - 908 mL H_2O

2. Wrap the membrane in Saran wrap, tape it to a blot paper and expose an x-ray film overnight at -70°C. Develop the film and identify the correct positively hybridizing E. coli transformants.

Grow bacterial clones in Terrific Broth (TB)

1. With a sterile bacteriological loop, pick one transformant (white) bacterial colony from the LB plate and add to 3 mL TB broth + 120 µL ampicillin (2.5 mg/mL stock).
   TB broth (per 1 liter diH_2O): 1.2% bacto-tryptone (w/v), 2.4% bacto-yeast extract (w/v), 0.4% glycerol (v/v); autoclave and add 100 mL of sterile solution of 0.17 M KH_2PO_4 and 0.72 MK_2HPO_4.
2. Grow the cells over night at 225 rpm and 37°C.

Glycerol stock from bacterial clones

1. To prepare 15% glycerol stock for long-term storage at -70°C, add 850 µL of cell suspension to 150 µL of ice-cold, sterile glycerol. Glycerol is already aliquotted into microfuge tubes. Keep microfuge tubes on ice.
2. Mix well and store at -70°C.

Grow up glycerol stock

1. Add 10 µL of glycerol stock to 3 mL TB broth + 120 µL ampicillin (2.5 mg/mL stock).
2. Grow the cells over night at 225 rpm and 37°C.
3. Purify plasmid DNA the following day.

Purification of plasmid DNA from E. coli

1. Pellet 1.5 mL of culture for 1 min at 12,000 rpm in a microcentrifuge. Remove the supernatant. (A total of 4.5 mL of culture can be spun down into one tube).
2. Resuspend the pellet in 200 µL of GTE (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) buffer by pipetting up and down.
3. Add 300 µL of 0.2 N NaOH/1% SDS (make fresh each time) buffer, mix by tube inversion, and incubate on ice for 5 min.
4. Add 300 µL of 3.0 M KOAC, pH 4.8, mix by tube inversion, and incubate on ice for 5 min.
5. Centrifuge the tube for 10 min at 12,000 rpm in a microcentrifuge tube at room temperature and transfer supernatant (approximately 700 µL) to a clean tube. Centrifuge the supernatant again for 10 min and transfer to a clean tube.
6. Add RNAase A (10 mg/mL stock) to a final concentration of 20 µg/mL. Incubate at 37°C for 20 min.

7. Do two $CHCl_3$ extraction using 400 μL per extraction. (The tube capacity is too small to use an equal volume of $CHCl_3$ but 1/2 vol works fine). Mix the phases by tube inversion. Centrifuge the tube for 1 min and remove the aqueous phase to a clean tube.
8. Precipitate the DNA by adding an equal vol of 100% isopropanol (~ 700 μL), pellet the DNA by centrifugation at 12,000 rpm for 15 min at room temperature, and remove the supernatant. Wash the pellet with 500 μL of 70% ethanol by inverting tube several times, then dry pellet briefly (5 min) under vacuum.
9. Dissolve the pellet in 32 μL diH_2O, and sequentially add 8.0 μL 4 M NaCl, mix, and 40 μL 13% PEG_{8000}. Mix well and incubate on ice for 1 h.
10. Centrifuge the tube at 12,000 rpm for 15 min at 4°C. Carefully remove the supernatant. The pellet will be translucent at this point and hard to see which is why a fixed angle rotor is preferable to a horizontal rotor. Rinse the pellet with 500 μL of 70% ethanol.
11. Dry the pellet under vacuum for 5 min and resuspend the DNA in 20 μL of deionized H_2O.

SeaPlaque agarose purification

1. Electrophorese digested plasmid DNA on 0.8% SeaPlaque agarose; include 1 kb λ DNA ladder (BRL Life Technologies. Inc.) and λ bacteriophage DNA cut with *Hind*III (λ/*Hind*III cut DNA).
2. Cut out the band of interest.
3. Melt the slice of agarose containing the band of interest at 65°C for 30 min; warm agarose diluent (0.2 M NaCl, 0.02 M Tris-HCl, pH 7.5, 0.002 M EDTA) at same time.
4. Dilute the molten slice to at least 0.3% agarose concentration with diluent and keep at 65oC for another 5 min.
5. Extract plasmid DNA 3 times with warm Tris-HCl saturated phenol, pH 7.0 (37°C).
6. 3 times ether extraction; evaporate ether on heating pad.
7. Ethanol precipitation:
    Add 1 μL of 1 μg/μL t-RNA
    Add 1/2 vol of 7.5 M ammonium acetate and 2 vol of 100% EtOH
    Keep on ice for 10 min (to overnight)
    Centrifuge for 10-20 min
    Wash pellet with 500 μL of 70% EtOH
    Dry under vacuum
    Dissolve pellet in 20 μL of sdH_2O and heat at 56°C for 10 min.

Cycle sequencing of PCR product or plasmid DNA

Use fmol sequencing kit from Promega and follow the instructions.

1. Purify PCR product with Qiagen PCR purification column; elute DNA with sterile, distilled water
2. Label four 0.5 mL microfuge tubes (G,A,T,C) for each set of sequencing reactions. Add 2 µL of the appropriate d/ddNTP mix to each tube.
3. For the sequencing reaction pipette:

template:	500 fmol
primer:	4 pmol
$[\alpha\text{-}^{35}S]dATP$:	6 µCi (~1 µL)
5x sequencing buffer (250 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$)	5 µL
Taq DNA polymerase	1 µL (5U/µL)
sdH_2O	to a total reaction volume of 16 µL.

4. Aliquot 4 µL of template/primer/polymerase mix into each of the d/ddNTP mix tubes.
5. Place tubes in PCR machine preheated to 95°C and cycle 30 times through following temperature profile:

95°C	2 min
95°C	30 sec
42°C	30 sec
70°C	1 min

6. Stop reaction by adding 3 µL of fmol Sequencing Stop Solution to each tube.

Polyacrylamide sequencing gel

1. For a 21 cm x 42 cm, 8% acrylamide sequencing gel, mix 33.6 g urea, 7.0 mL of 10x TBE (0.9 M Tris-borate, pH 8.3, 0.02 M EDTA, pH 8.0), 10.5 mL of 40% 19:1 acrylamide:bis-acrylamide, and diH_2O up to 70 mL. Dissolve the reagents on a heating/stirring plate at low heat.
2. While the reagents are dissolving, clean the glass plates of the sequencing unit (BIO-RAD Sequi-Gen^{TM} very well with a paste of detergent. Rinse glass plates in diH_2O and 70% EtOH. Dry them well, assemble sequencing gel unit and place into a casting tray. (Periodically, apply a few drops of Rainex to the plate with the buffer tank to prevent the gel from sticking to it).
3. Vacuum-filtrate the sequencing gel solution through a Whatman #3 filter paper and degas it.
4. To pour the base, measure 30 mL of the gel solution, add 375 µL of 10% ammonium persulfate (APS) and 80 µL of 100% N, N, N', N' - tetramethylethylenediamine (TEMED), mix and pour rapidly. The gel will polymerize in about 1 min.
5. To pour the gel between the plates, use the remaining gel solution (40 mL), add 280 µL of APS and 10 µL of TEMED, mix well and pipette between the two glass plates. Be careful not to get any bubbles.
6. Clamp a sharkstooth comb in inverted position (Teeth pointing away from the gel) between the glass plates and let gel polymerize (about 1 h).

7. After gel polymerization, remove sharkstooth comb and rinse the unit briefly in water to remove the crystallized urea. Mount the gel unit on the electrophoresis apparatus.
8. Make 1,500 mL of 1 x TBE, pour 1 x TBE into the buffer tank of one glass plate and the buffer tank at the bottom.
9. Rinse the space between the two glass plates and polymerized gel very well with a syringe filled with buffer to remove bits of urea. Insert the sharkstooth comb with the teeth pointing into the gel.
10. Rinse the bottom of the gel unit (standing in the buffer tank filled with buffer) to remove any trapped air bubbles (which would affect the flow of current).
11. Pre-run the gel at 1,800 V for at least 30 min to heat the gel.
12. Heat the sequencing reactions to 70°C for 2 min just prior to loading them onto the gel. With a flat pipette tip, load 3.5 µL of each reaction into one of the wells formed by the sharkstooth comb.
13. Run the gel for 3 h at 1,800 V, then load another round of sequencing reactions and run for an additional 3 h. Repeat one more time.
14. Turn off the power, pour out the buffer (collect the buffer from the bottom tank in a receptacle for radioactive waste), and disassemble the unit. Pull the glass plate with the buffer tank away from the other plate; the gel should stick to the other plate. Remove the spacers.
15. Fix the gel (still supported by the other glass plate) in 5% acetic acid, 15% EtOH for 30 min.
16. Lift the gel (supported by the glass plate) from the fixing solution, drain off residual liquid and blot it onto a sheet of Whatman 3MM paper. Put it onto a gel dryer and cover it with a piece of Saran wrap, making sure no bubbles form between the gel and the Saran wrap. Dry it under vacuum for 1 h at 80°C. Collect the radioactive fixing solution in a proper container.
17. Expose the gel to Kodak diagnostic x-ray film at -70°C for 2-3 d.

16S rRNA gene sequence of T. solenopsae

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   1  GAATTCCACC AGGTTGATTC TGCCTGGTAT GTGTGCTAGC GTCAAGGATT
  51  TAGCCATGCA TGCTTACGAA CCCACGTGGG GAGTGGCGGA TAGCTCAGTA
 101  ATACAGTTAT AACATAATCT ACATAAATGG ATAACCTTGT CAAGATAAGG
 151  CTAATACAGT AAAGATGTTA GAAGCATGAA AGCGGAGCAT CATTGTAGGA
 201  TTGGTTTCTG ACCTATCAGT TAGTATGTTT TGTAAGGGAG AACATAGACT
 251  ATGACGGGTA ACGGGGGATG CACGTCTGAT ACCGGAGAGG AAGCCTTAGA
 301  AACCGCTTTC ACGTCCAAGG ATGGCAGCAG GCGCGAAACT TACCCAATTA
 351  TTGTATTGAT AGAGGTAGTT ATGACGCATG TTAAGATTTT AAATTGAAAC
 401  TTCATTAAAG ATGGTTAAGC GACTGGAGGG CAAGTCTGGT GCCAGCAGCC
 451  GCGGTAATTC CAGCTCCAGT AGTGCATATA CATGCTGTAG TTAGAAAGTT
 501  TGTAGCCAGT TTATGGATTG TTTTTGATAA TAGTTATTCT CCAAAAGAGC
 551  TAATTTTAAC TAATTCATAA AAATAGAAGC GGATGAAGGT AATTGTATTC
 601  ACCAGCAAGA GGTAAAATTT GATGACCTGG TGAGGACATT CCGAGGCGAA
 651  AGCGATTGCC TAGTACGTTT TTGATGGTAA AGAACGTAAG CCGGAGGATC
 701  AAAGATGATT AGATACCGTT GTAGTTCCGG CCGTAAATTA TGCCAACTTG
 751  CATCTTGTTA TTTATACAAG GAGCATAGAG AAATTAAGAG TTTTTGGGCT
 801  CTAGGGATAG TAATCCGGCA ACGGACAAAC TTAAAGAAAT TGGCGGAAGG
 851  ACACCACAAG GAGTGGATTA TACGGCTTAA TTTGACTCAA CGCGGGAAAA
 901  CTTACCAGGG CCTATGTATA AGAGAAAGTT AACATTGTAT GTATACTTGA
 951  TTGTACTTTG AGTGGTGCAT GGCCGTTTTC AACACGTGGG GTGACTTGTC
1001  AGGTTTATTC CGGTAACGTG TGATGTGCAG TATGCAACTA ACTAATGTTG
1051  TGAGACTTCT TGCGGTAAGC TTGATGAAGA GGCGCTATAA CAGGTCAGTG
1101  ATGCCCTTAG ATGTTCTGGG CTGCACGTGT AATACAGTGG GTATTTCAAT
1151  ATTTAATAGA AGTAAATTTA CCCGAGACAG GGATCATGCT TTGTAAGAAG
1201  CTTGTGAACA TGGAATTCCT AGTAATCGCT GCTCACTAAG TAACGATGAA
1251  TAAGTCCCTG TTCTTTGCAC ACACCGCCCG TCGCTATCTG AGATGGATGA
1301  CTTTATAAAG ATGCTGCTGT GAAGAGGCAT TTGTGTAAGG TCAACTAGAT
1351  TAGATATAAG TCGTAACAAG GTAACCGGAT CC
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Partial 16S rRNA gene sequence of Thelohania sp.

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   1  CGAACCCATG TGGGGAGTGG CGGATAGCTC AGTAATACAG TTATAACATA
  51  ATCTACATAA ATGGATAACC TTGTCAAGAT AAGGCTAATA CAGTAAAGAT
 101  GTTAGAAGCA TGAAAGCGGA GCATCAATGT AGCGTTGGTT TCTGACCTAT
 151  CAGTTAGTAT GTTTTGTAAG GGAGAACATA GACTATGACG GGTAACGGGG
 201  GATGCACGTC TGATACCGGA GAGGAAGCCT TAGAAACCGC TTTCACGTCC
 251  AAGGATGGCA GCAGGCGCGA AACTTACCCA ATTATTGTAT TGATAGAGGT
 301  AGTTATGACG CATGTTAAGA TTTTAAATTG AAACTTCATT AAAGATAGAT
 351  AAGCGACTGG AGGGCAAGTC TGGTGCCAGC AGCCGCGGTA ATTCCAGCTC
 401  CAGTAGTGCA TATACATGCT GTAGTTAGAA AGTTTGTAGC CAGTTTATGG
 451  ATTGTTTTTG ATAATAGTTA TTCTCCAAAA GAGCTAATTT TAACTAATTC
 501  ATAAAAATAG AAGCGGATGA AGGTAATTGT ATTCACCAGC AAGAGGTAAA
 551  ATTTGATGAC CTGGTGAGGA CATTCAGAGG CGAAAGCGAT TGCCTAGTAC
 601  ATTTTTGATG GTAAAGAACG TAAGCCGGAG GATCAAAGAT GATTAGATAC
 651  CGTTGTAGTT CCGGCCGTAA ATTATGCCAA CTTGCATTTT GTTATTTATA
 701  CAAGGAGCAT AGAGAAATTA AGAGTTTTTG GGCTCTAGGG ATAGTAATCC
 751  GGCAACGGAC AAACTTAAAG AAATTGGCGG AAGGACACCA CAAGGAGTGG
 801  ATTATACGGC TTAATTTGAC TCAACGCGGG AAAACTTACC AGGGCCTATG
 851  TATAAGAGAA AGTTAACATT GTATGTATAC TTGATTGTAC TTTGAGTGGT
 901  GCATGGCCGT TTTCAACACG TGGGGTGACT TGTCAGGTTT ATTCCGGTAA
 951  CGTGTGATGT GCAGTATGCA ACTAATGTTG TGAGACTTCT TGCGGTAAGC
1001  TTGATGAAGA GGCGCTATAA CAGGTCAGTG ATGCCCTTAG ATGTTCTGGG
1051  CTGCACGTGT AATACAGTGG GTATTTCAAT ATTTAATAGG AGTAAATTTA
1101  CCCGAGACAG GGATCATGCT TTGTAAGAAG
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Partial 16S rRNA gene sequence of Vairimorpha sp.

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   1  TCAGAGATTA AGCCATGCAA GCCAGCGAAG ATTACGGAGC GGCGTACGGC
  51  TCAGTAGGAC AGGGAAATCT AGCCACGAAG GAGGATAACC ACGGTAAGCT
 101  GTGGCTAAAA CGAGCGTGGG TGAGTTCTTG GCCTATCAGC TAGTCGGTAC
 151  GGTAAGGGCG TACCGAGGCA ATAACGGGTA ACGGGGAATC GGGGTTCGAT
 201  TCCGGAGAGG AAGCCTGAGA AACGGCTACC ACGTCCAAGG AAGGCAGCAG
 251  GCGCGGAAAT TACCCACTTG GAGGACCAGA GGTAGTTATG GGGCGTAAAG
 301  ATGAGAAAAG TGTAAAAAGC TTTTTGAATG CGACTGGAGG GCAAGTCTGG
 351  TGCCAGCAGC CGCGGTAATT CCAGCTCCAG GAGCTTCTGT GTGAGTTGCT
 401  GCGGTTAAAA AGTGCGTAGT CGGGGAGCAG GCCAGCAGAA AAGGTGGGGA
 451  ATCACGCCTA GCATAGTGCA GGAACTGGGA CCTAGGGACC GGAGAGGGGC
 501  AACCTAATTC TTGGGCGAGG GGTGAAAACT GCTGACCCTG AGAGGAGGAA
 551  CAGAGGCGAA GGCGGTTGTC CGGGACGGGT CTGACGATCA AGTACGTGAG
 601  CAGGAGGATC AAAGACGATT AGACACCGTC GTAGTTCCTG CAGTAAACGA
 651  TGCCGACGGG GCAGCAGGGG AACTTGTTGC CTGAGGGAAA CCAAGTGTAC
 701  GGGCTCCGGG GATAGTACGG GCGCAAGCTT GAAACTTAAA GAAATTGACG
 751  GAAGGACACC ACAAGGAGTG GAGTGTGCGG GTTAATTTGA CTCAACGCGG
 801  GACAACTTAC CGGGGCAGGC GACGAGAAGC GAAGGATGAT GAAGAGATTC
 851  ACAGACTGAT TGCGTCGCGT GTGGTGCATG GCCGTTTTTA ACACGTGGGG
 901  TGACTTGTCA GGTTAAATCC GATAACGCGT GAGACCCTGT GTAGATGGAA
 951  ATACGACGGG ACATGGCAAG TGTCAGGAAG AGCGGGTCGA TAACAGGTCT
1001  GTGATGCCCG CAGATGTTCC GGGCGCCACG CGCACTACAT TGGACGGCGA
1051  TATATGAAAA TGAGGAGCCG TCCGTGGTTG GGATTGACGC TTGTAATTGC
1101  GTCATGAACG TGGAATTCCT AGTAGTGGGC AGTCATTAAC TGCACGCGAA
1151  TGAGTCCCTG TTCTTTGTAC ACACCGCCCG TCGTTATCTA AGATGGAAGT
1201  GCGGATGAGG TCGGTACGGC CGGACGAATC TGTGCTTGTA GATTGGATAC
1251  AA
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Partial 16S rRNA sequence of A. penaei

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   1  ACTTTTAACT AACCTTTTGT ACTAATAATT AAGGGAAACT GTAATTAAAA
  51  ATCATGAGGA TGTGAGGTAG ACCTATTAGC TAGTTGGTTG TGTAAAGGAC
 101  TACCAAGGCT ATAATGGGTA ACGGAGATTT AGTGATCGAA ACCGGAGATG
 151  GAAGCTGAGA AACGGTTCCA ATGTCCAAGG ATAGCAGCAG GCGCGAAAAT
 201  TGCACACTCT TTAATGGGGA TGCAGTTATG AGGTATGACA GAAAGGGTTA
 251  TCAATAAATA AGATGACGTA AAGCTATTAG AGGGAAAGTT TGGTGCCAGC
 301  AGCCGCGGTA ATACCAACTC TAAGAGTCTC TATGCGAGTT GCTGCAGTTA
 351  AAAAGTCCGT AGTCTTTACG TAATTAAAAA TGAATGATCA AGTTTCATAT
 401  TTTTACGTTT ATATATGAGA CGGATTGGGA GCATAGTATA ACTGGGTTAA
 451  GAATGAAATC TCACTACCCT AGTTGGACTA TCAGAAGCGA AAGCGATGCT
 501  CTAATACGTA CTTTTAGATA AAGGACGAAG GCTAGAGTAG CGAAAGGGAT
 551  TAGATACCCC TGTAGTTCTA GCAGTAAACT ATGCCGACAG AATGTTAGAT
 601  ATATTTCTAG TGTTCAAGGG AAACCTTAAG TGATCGGGCT CTGGGGAGAG
 651  TATGCTCGCA AGTGTGAAAA TTAAACGAAA TTGACGGAGT TACACCACAA
 701  GGAGTGGATT GTGCGGCTTA ATTTGACTCA ACGCGAGGAA TTTTACCAGG
 751  GCTGAATATA TTTGAGATTG AATACATGAA ATATATTTGA GTGGTGCATG
 801  GTCGTTGTAA ACTCATGGAT TGATCTTAAG TTCAACTGCT AAAATGGGTG
 851  AGACTTTCAT AAACAGCTAT CTAACAGGTA GAGGAAGGGG AAGGCGATAA
 901  CAGATCCGTG ATGCCCTCAG ATGTCCTGGG CTGCACGCGC AATACATTAT
 951  GTATATTTCT TATAAATAGA TACTACATAT TGGGGAATTG ACTTTTGTAA
1001  ATAAGTCATG AACTTGGAAT TCCTAGTAAT AATGATTCAT CAAGTCATTG
1051  TGAATGTGTC CCTGTAGCTT GTACACACCG CCCGTCACTG TCTCAGATGG
1101  TTGATGAGAT G
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BIOGRAPHICAL SKETCH

Bettina Angela Moser was born on 4 September 1961 in Landstuhl/Pfalz, then West-Germany. Following graduation from the "Burggymnasium" , Kaiserslautern, 1980, she studied Cultures of the Middle East at the "Universität des Saarlandes" in Saarbrücken from 1980-82. She then went to the "Freie Universität" in Berlin where she majored in biology and Persian language. She received the "Vordiplom" (equivalent of B.S.) in biology in 1984.

In 1986 she came to the University of Florida as a non-degree graduate exchange student for one year and took courses in the Entomology and Nematology Department. She decided to stay and pursue her M.S. in that department which was awarded in 1989. After two years as a research assistant working on insect-pathogenic fungi, she started her Ph.D. project on microsporidia pathogenic to imported fire ants under the direction of Drs. Richard Patterson and James Becnel.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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